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Express Mailing Label No. EL951599336US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 5,275,813

Issued: January 4, 1994

To: Janet K. Yamamoto et al.

Assignee: The Regents of the University of California

For: METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

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MAY 15 2002

OFFICE OF PETITIONS

ATTN: BOX PATENT EXT.

Commissioner for Patents  
Washington, D.C. 20231

Sir:

**APPLICATION FOR EXTENSION OF PATENT  
TERM UNDER 35 U.S.C. §156**

Applicant, The Regents of the University of California, represents that it is the Assignee of the entire interest in and to United States Patent No. 5,275,813 (assignment recorded in the United States Patent and Trademark Office at Reel 5796, Frame 495) granted to Janet K. Yamamoto and Niels C. Pedersen on the 4th day of January, 1994, for Methods and Compositions for Vaccinating Against Feline Immunodeficiency Virus. By the Power of Attorney enclosed herein (Attachment A), Applicant appoints Kevin L. Bastian of Townsend and Townsend and Crew, Customer Number 20350, as attorney for Applicant with regard to this application for extension of

the term of U.S. Patent 5,275,813 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

**Information Required Under 37 C.F.R. §1.740**

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. §156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. §1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented in a format which follows the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

- (1) The approved product is the Fel-O-Vax® FIV vaccine (VS Code No. 15A5.21). The vaccine is a feline immunodeficiency virus vaccine comprising inactivated subtype A and subtype D FIV whole virus.
- (2) The approved product was subject to regulatory review under the Virus-Serum-Toxin Act (21 U.S.C. §§ 151-159) and corresponding regulations (9 C.F.R. §102).
- (3) The approved product Fel-O-Vax® FIV vaccine received permission for commercial marketing or use under the Virus-Serum-Toxin Act on March 14, 2002. A copy of the approval letter and the U.S. Veterinary Biological Product License are attached (Attachment B).
- (4) The active ingredients in Fel-O-Vax® FIV vaccine include inactivated subtype A and subtype D FIV whole virus, which, on information and belief, have not

been approved for commercial marketing or use under the Virus-Serum-Toxin Act prior to approval by the Department of Agriculture on March 14, 2002.

(5) This application for extension of patent term under 35 U.S.C. §156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. §1.720(f), said period will expire on May 13, 2002.

(6) The complete identification of the patent for which a term extension is being sought is as follows:

Inventors: Janet K. Yamamoto and Niels C. Pedersen

Patent No.: 5,275,813

Issue Date: January 4, 1994

Expiration Date: January 4, 2011

(7) A true copy of the patent is attached (Attachment C).

(8) No certificate of correction, disclaimer or reexamination certificate has been iss

ued on this patent. A copy of a record of maintenance fee payments under 35 U.S.C. §41(b) is attached (Attachment D).

(9) U.S. Patent 5,275,813 claims a vaccine and a method of administering a vaccine. Claims 1-3 read on the Fel-O-Vax® FIV vaccine, or on its method of use.

Claims 1 and 3 are directed to a vaccine against feline immunodeficiency virus infection comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, and reads on the approved product because the approved product is a feline immunodeficiency virus vaccine that contains inactivated subtype A and subtype D FIV whole virus.

Claim 2 is directed to a method of administering the vaccine of claim 1 to a cat and reads on a method of using the approved product for the reasons noted above.

(10) The relevant dates and information pursuant to 35 U.S.C. §156(g) to enable the Secretary of Agriculture to determine the applicable regulatory review period are as follows:

The "date the authority to prepare an experimental biological product under the Virus-Serum-Toxin Act became effective", as stated in 37 C.F.R. §1.740(a)(10)(iii), is considered to be August 28, 1991. We have used the date of August 28, 1991, which is the date an Application for United States Veterinary Biological Product License (VS 1A55.20) was submitted to USDA including immunogenicity study results and challenge model development results involving a strain contained in the approved product. The original work was done by Solvay's U.S.-based animal health group that was acquired by American Home Products Corporation (AHP) effective February 28, 1997, as part of its acquisition of Solvay's global animal health business and assets. AHP became Wyeth effective March 11, 2002. Fort Dodge Laboratories is a division or wholly owned subsidiary of Wyeth. AHP, now Wyeth, is a licensee under U.S. Patent No. 5,275,813.

A U.S. Veterinary Biological Product License application for Fel-O-Fax® FIV vaccine was submitted on May 4, 1998, and such license (V.S. Code No. 15A5.21) was issued on March 14, 2002.

(11) A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to Fel-O-Vax® FIV vaccine and the dates applicable to these significant activities are set forth in a chronology of events in Attachment E.

(12)(i) Applicant is of the opinion that U.S. Patent 5,275,813 is eligible for extension of the patent term under 35 U.S.C. §156 because it satisfies all requirements for such extension as follows:

- (a) 35 U.S.C. §156(a) - U.S. Patent 5,275,813 claims a method for using the Fel-O-Vax® FIV vaccine, as well as the vaccine itself.
- (b) 35 U.S.C. §156(a)(1) - U.S. Patent 5,275,813 has not expired before submission of this application.
- (c) 35 U.S.C. §156(a)(2) - The term of U.S. Patent 5,275,813 has never been extended under 35 U.S.C. §156(e)(1).
- (d) 35 U.S.C. §156(a)(3) - The application for extension is submitted by the agent of the owner of record of the patent in accordance with the requirements of paragraphs (1) through (4) of 35 U.S.C. §156(d) and the rules of the Patent and Trademark Office.
- (e) 35 U.S.C. §156(a)(4) - The Fel-O-Vax® FIV vaccine has been subjected to a regulatory review period before its commercial marketing or use.
- (f) 35 U.S.C. §156(a)(5)(A) - The commercial marketing or use of the Fel-O-Vax® FIV vaccine after the regulatory review period is the first permitted commercial marketing or use under the Virus-Serum-Toxin Act (21 U.S.C. §§151-159) under which such regulatory review occurred.
- (g) 35 U.S.C. §156(c)(4) - No other patent has been extended for the same regulatory review period for the Fel-O-Vax® FIV vaccine.

(12)(ii) The length of the extension of patent term of U.S. Patent 5,275,813 claimed by Applicant is that period authorized by 35 U.S.C. §156(c), which has been calculated to be 5 years. The length of the extension was determined pursuant to 37 C.F.R. §1.779 as follows:

(a) The regulatory review period under 35 U.S.C. §156(g)(5)(B) began on August 28, 1991, and ended March 14, 2002, which is a total of 3853 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. §156(g)(5)(B)(i) began on August 28, 1991, and ended on May 4, 1998, which is 2442 days; and

(2) The period of review under 35 U.S.C. §156(g)(5)(B)(ii) began on May 4, 1998, and ended on March 14, 2002, which is a total of 1411 days.

(b) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in subparagraph 12(ii)(a) above (3853 days) less:

(1) The number of days in the regulatory review period which were on or before the date on which the patent issued (January 4, 1994), which is 860 days; and

(2) The number of days during which applicant did not act with due diligence, which is zero (0) days; and

(3) One-half the number of days determined in sub-paragraph (12)(ii)(a)(1) above less the number of days in 12(ii)(b)(1) (one-half of 1582), which is 791 days;

(c) The number of days as determined in sub-paragraph (12)(ii)(b) (2201) when added to the original term of the patent (January 4, 2011) would result in the date of January 14, 2017.

(d) Fourteen (14) years when added to the date of issuance of a license under the Virus-Serum-Toxin Act (March 14, 2002) would result in the date of March 14, 2016;

(e) The earlier date as determined in sub-paragraphs (12)(ii)(c) and (12)(ii)(d) is March 14, 2016;

(f) Since the patent was issued after November 16, 1988, and since no request for the authority to prepare an experimental biological product under the Virus-Serum-Toxin Act was submitted before November 16, 1988, the period of extension may not exceed five years from the original expiration date of January 4, 2011. Five years when added to the original expiration date of the patent would result in the date of January 4, 2016.

(g) The earlier date as determined by sub-paragraphs (12)(ii)(e) and (12)(ii)(f) is January 4, 2016.

(13) Applicant acknowledges a duty to disclose to the Director of Patents and Trademarks and the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought.

(14) The Director is authorized to charge Deposit Account No. 20-1430 in the amount of \$1,120.00, the fee due for receiving and acting upon this application, as well as any additional fees required by this application

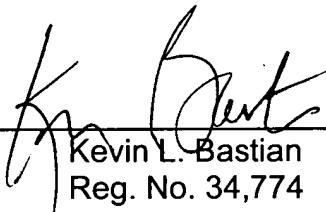
(15) All correspondence and inquiries may be directed to the undersigned, whose address, telephone number and fax number are as follows:

Kevin L. Bastian  
Townsend & Townsend & Crew  
Two Embarcadero Center, 8<sup>th</sup> Floor  
San Francisco, CA 94111-3834

(16) Enclosed is a certification that the application for extension of patent term under 35 U.S.C. §156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof (Attachment F).

Respectfully submitted,

By: \_\_\_\_\_

  
Kevin L. Bastian  
Reg. No. 34,774

Date: May 10, 2002

Attachments:

Authorization to Charge Deposit Account No. 20-1430 for \$1,120.00  
Power of Attorney (Attachment A)  
Approval Letter and License (Attachment B)  
U.S. Patent 5,275,813 (Attachment C)  
Receipt of Maintenance Fee Payments (Attachment D)  
Chronology of Regulatory Review Period (Attachment E)  
Certification of Copies of Application Papers (Attachment F)

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## ATTACHMENT A

Please type a plus sign (+) inside this box → 

PTO/SB/81 (02-01)

Approved for use through 10/31/2002. OMB 0651-0055  
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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**POWER OF ATTORNEY OR  
AUTHORIZATION OF AGENT**

MAY 10 2002

SIGNED  
PATENT & TRADEMARK OFFICE

Application Number	07/739,014 (US Patent No. 5,275,813)
Filing Date	07/31/91 (Issue Date: 01/04/94)
First Named Inventor	Janet K. Yamamoto
Title	Methods and Compositions for Vaccinating Against Feline Immunodeficiency Virus
Group Art Unit	1813
Examiner Name	Barnd, D.
Attorney Docket Number	02307U-023730US

I hereby appoint:

 Practitioners at Customer Number

OR

 Practitioner(s) named below:

20350

Place Customer  
Number Bar Code  
Label here

Name	Registration Number

as my/our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

Please change the correspondence address for the above-identified application to:

 The above-mentioned Customer Number.

OR

 Practitioners at Customer Number
 →
 Firm or Individual Name

Address

Address

City

State

ZIP

Country

Telephone

Fax

I am the:

 Applicant/Inventor. Assignee of record of the entire interest. See 37 CFR 3.71.

Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

## SIGNATURE of Applicant or Assignee of Record

Name Candace L. VoelkerSignature Candace L. VoelkerDate 5/8/02

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below\*.

 \*Total of \_\_\_\_\_ forms are submitted.

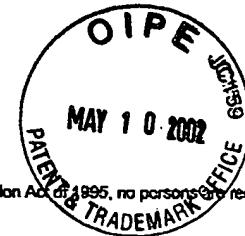
Burden Hour Statement: This form is estimated to take 3 minutes to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.  
SF 1344477 v1

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MAY 15 2002

OFFICE OF PETITIONS

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PTO/SB/95 (08-00)

Approved for use through 10/31/2002, OMB 0651-0031  
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE  
number.

Attorney Docket No. 02307U-023730US

**STATEMENT UNDER 37 CFR 3.73(b)**Applicant/Patent Owner: The Regents of the University of CaliforniaApplication No./Patent No.: (07/739,014) US Patent No. 5,275,813 Filed/Issue Date: (July 31, 1991) Issued: January 4, 1994Entitled: Methods and Compositions for Vaccinating Against Feline ImmunodeficiencyThe Regents of the University of California, a university(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency,  
etc.)

states that it is:

- the assignee of the entire right, title, and interest; or
- an assignee of less than the entire right, title and interest.  
The extent (by, percentage) of its ownership interest is \_\_\_\_\_ %

in the patent application/patent identified above by virtue of either:

A.  An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 5796, Frame 0494, 0495, 0496, or for which a copy thereof is attached.

OR

B.  A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as shown below:

1. From: \_\_\_\_\_ To: \_\_\_\_\_  
The document was recorded in the United States Patent and Trademark Office at Reel \_\_\_\_\_, Frame \_\_\_\_\_, or for which a copy thereof is attached.

2. From: \_\_\_\_\_ To: \_\_\_\_\_  
The document was recorded in the United States Patent and Trademark Office at Reel \_\_\_\_\_, Frame \_\_\_\_\_, or for which a copy thereof is attached.

3. From: \_\_\_\_\_ To: \_\_\_\_\_  
The document was recorded in the United States Patent and Trademark Office at Reel \_\_\_\_\_, Frame \_\_\_\_\_, or for which a copy thereof is attached.

Additional documents in the chain of title are listed on a supplemental sheet.

Copies of assignments or other documents in the chain of title are attached.

**[NOTE:** A separate copy (i.e., the original assignment document or a true copy of the original document) must be submitted to Assignment Division in accordance with 37 CFR Part 3, if the assignment is to be recorded in the records of the USPTO. See MPEP 302.8]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

5/8/02  
Date

Candace L. Voelker

Typed or printed name

Candace L. Voelker  
Signature

Associate Director

Title

**Burden Hour Statement:** This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.



ATTACHMENT B

**OVERNIGHT MAIL**  
March 14, 2002

United States  
Department of  
Agriculture

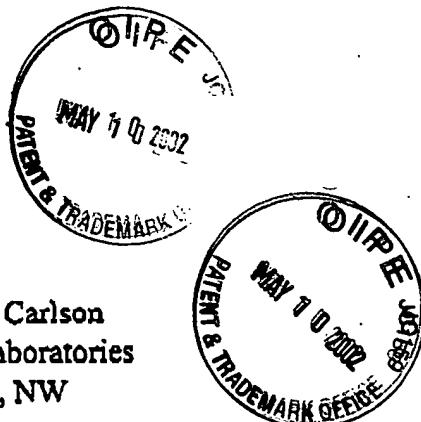
Marketing and  
Regulatory  
Programs

Animal and Plant  
Health Inspection  
Service

Veterinary Services

Center for Veterinary  
Biologics  
Suite 104  
510 South 17th Street  
Ames, IA 50010  
(515) 232-5785  
FAX (515) 232-7120

Federal Relay Service  
(Voice/TTY/ASCII/  
Spanish)  
1-800-877-8339



Ms. Madonna Carlson  
Fort Dodge Laboratories  
800 5th Street, NW  
P.O. Box 518  
Fort Dodge, IA 50501

Dear Ms. Carlson:

Enclosed is a new United States Veterinary Biological Product License issued this date to American Home Products Corporation, Establishment No. 112, authorizing production of the following:

Feline Immunodeficiency Virus Vaccine, Killed Virus, Code 15A5.21

Please note the restrictions under which this license is issued.

This U.S. Veterinary Biological Product License does not constitute a patent license. If this product or technology used in the manufacture of this product has been patented or is pending patent, the licensee should obtain a patent license from the patent owner.

If this license does not agree with your records, please return it to this office with your comments.

Sincerely,

Richard E. Hill, Jr., D.V.M.  
Director  
Center for Veterinary Biologics

Enclosure

cc: Bio Regulatory Affairs  
FILE: VS Code 15A5.21  
Product License Book

(see Bio Reg Notice #13 for  
distribution list)

**RECEIVED**

MAR 15 2002

BIOLOGICAL  
REGULATORY AFFAIRS



# United States Department of Agriculture

## UNITED STATES VETERINARY BIOLOGICAL PRODUCT LICENSE

Washington, D.C.

This is to certify that, pursuant to the terms of the Act of Congress approved March 4, 1913 (37 Stat. 832), governing the preparation, sale, barter, exchange, shipment, and importation of viruses, serums, toxins, and analogous products intended for use in the treatment of domestic animals, the person holding United States Veterinary Biologics Establishment License No. 112 is authorized to prepare in the facilities designated in the establishment license:

### FELINE IMMUNODEFICIENCY VIRUS VACCINE

Killed Virus

Code 15A5.21

Preparation shall be in accordance with the provisions of the Act, the regulations made thereunder, and additional restrictions or requirements when listed below.

1. For use by or under the supervision of a veterinarian.
2. Marketing and promotional materials must be submitted to the Center for Veterinary Biologics for review and approval prior to use.

This license is subject to termination as provided in the regulations made under the authority contained in said Act, and to suspension or revocation if the licensee violates or fails to comply with said Act or the regulations made thereunder.

March 14, 2002

Date



Director, Center for Veterinary Biologics  
Animal and Plant Health Inspection Service



ATTACHMENT F ]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 5,275,813 )  
Issued: January 4, 1994 )  
To: Janet K. Yamamoto et al. )  
Assignee: The Regents of the University of )  
California )  
For: METHODS AND COMPOSITIONS FOR )  
VACCINATING AGAINST FELINE )  
IMMUNODEFICIENCY VIRUS )

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

**CERTIFICATION**

I hereby certify that this accompanying application for extension of the term of U.S. Patent 5,275,813 under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof.

Respectfully submitted,

Date: May 10, 2002

By: *Kevin L. Bastian*  
Kevin L. Bastian  
Reg. No. 34,774

Please type a plus sign (+) inside this box → +

Approved for use through 10/31/2002. OMB 0651-0031

U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

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**TRANSMITTAL  
FORM**

MAY 10 2002

(to be used for all correspondence after initial filing)  
Express Mail Label No. EL951599336US

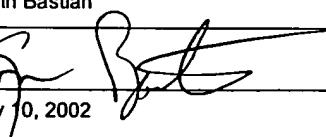
Application Number	07/739,014 (US Patent 5,275,813)
Filing Date	07/31/91 (Issued: 01/04/94)
First Named Inventor	Yamamoto, Janet K.
Examiner Name	
Attorney Docket Number	02307U-023730US

Total Number of Pages in This Submission

**ENCLOSURES (check all that apply)**

<input checked="" type="checkbox"/> Fee Transmittal Form	<input type="checkbox"/> Assignment Papers (for an Application)	<input type="checkbox"/> After Allowance Communication to Group
<input type="checkbox"/> Fee Attached	<input type="checkbox"/> Drawing(s)	<input type="checkbox"/> Appeal Communication to Board of Appeals and Interferences
<input type="checkbox"/> Amendment / Response	<input type="checkbox"/> Licensing-related Papers	<input type="checkbox"/> Appeal Communication to Group (Appeal Notice, Brief, Reply Brief)
<input type="checkbox"/> After Final	<input type="checkbox"/> Petition Routing Slip (PTO/SB/69) and Accompanying Petition	<input type="checkbox"/> Proprietary Information
<input type="checkbox"/> Affidavits/declaration(s)	<input type="checkbox"/> Petition to Convert to a Provisional Application	<input type="checkbox"/> Status Letter
<input type="checkbox"/> Extension of Time Request	<input type="checkbox"/> Power of Attorney, Revocation Change of Correspondence Address	<input checked="" type="checkbox"/> Other Enclosure(s) (please identify below):
<input type="checkbox"/> Express Abandonment Request	<input type="checkbox"/> Terminal Disclaimer	Application for Extension of Patent Term Under 35 U.S.C. §156 (10 pgs.); Attachment A: Executed Power of Attorney (1 pg.) and Executed Statement Under 37 CFR 3.73(b) (1 pg.); Attachment B: Approval Letter and License ( 2 pgs.); Attachment C: US Patent 5,275,813 (24 pgs.); Attachment D: Receipt of Maintenance Fee Payments (1 pg.); Attachment E: Chronology of Regulatory Review Period (4 pgs.); Attachment F: Certification of Copies of Application Papers (1 pg. + 2 pgs., copies); Express Mailing (Label No. EL951599336US); One Return Postcard
<input type="checkbox"/> Information Disclosure Statement	<input type="checkbox"/> Request for Refund	
<input type="checkbox"/> Certified Copy of Priority Document(s)	<input type="checkbox"/> CD, Number of CD(s)	
<input type="checkbox"/> Response to Missing Parts/ Incomplete Application		
<input type="checkbox"/> Response to Missing Parts under 37 CFR 1.52 or 1.53		
Remarks		The Commissioner is authorized to charge any additional fees to Deposit Account 20-1430.
Authorization to Charge Deposit Account No. 20-1430 in the amount of \$1,120.00 for fee due in this filing.		

**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT**

Firm and Individual name	Townsend and Townsend and Crew LLP Kevin Bastian Reg. No. 34,774	
Signature		
Date	May 10, 2002	

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# FEE TRANSMISSION for FY 2001

Patent fees are subject to annual revision.



TOTAL AMOUNT OF PAYMENT (\$ 1120)

Complete if Known	
Application Number	07/739,014 (US Patent No. 5,275,813)
Filing Date	07/31/91 (Issue Date: 01/04/94)
First Named Inventor	Yamamoto, Janet K.
Examiner Name	
Group Art Unit	

Attorney Docket No. 02307U-023730US

## METHOD OF PAYMENT (check all that apply)

Check  Credit Card  MoneyOrder  Other  None  
 Deposit Account:

Deposit Account Number

20-1430

Deposit Account Name

Townsend and Townsend and Crew LLP

The Commissioner is authorized to: (check all that apply)

- Charge fee(s) indicated below  Credit any overpayments  
 Charge any additional fee(s) during the pendency of this application  
 Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

## FEE CALCULATION

## 1. BASIC FILING FEE

Large Entity | Small Entity

Fee Code	Fee (\$)	Fee Code	Fee (\$)	Fee Description	Fee Paid
101	740	201	370	Utility filing fee	
106	330	206	165	Design filing fee	
107	510	207	255	Plant filing fee	
108	740	208	370	Reissue filing fee	
114	160	214	80	Provisional filing fee	

SUBTOTAL (1)

(\$)

## 2. EXTRA CLAIM FEES

Total Claims

-20\*\*

=

Extra Claims

X

Fee from below

=

Fee Paid

Independent Claims

-3\*\*

=

Extra Claims

X

Fee from below

=

Fee Paid

Multiple Dependent

X

=

Fee Paid

Large Entity | Small Entity

Fee Code	Fee (\$)	Fee Code	Fee (\$)	Fee Description	Fee Paid
103	18	203	9	Claims in excess of 20	
102	84	202	42	Independent claims in excess of 3	
104	280	204	140	Multiple dependent claim, if not paid	
109	84	209	42	" Reissue independent claims over original patent	
110	18	210	9	" Reissue claims in excess of 20 and over original patent	

SUBTOTAL (2)

(\$)

\*\* or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

## 3. ADDITIONAL FEES

Large Entity	Fee Code	Fee (\$)	Small Entity	Fee Code	Fee (\$)	Fee Description	Fee Paid
	105	130		205	65	Surcharge - late filing fee or oath	
	127	50		227	25	Surcharge - late provisional filing fee or cover sheet	
	139	130		139	130	Non-English specification	
	147	2,520		147	2,520	For filing a request for reexamination	
	112	920*		112	920*	Requesting publication of SIR prior to Examiner action	
	113	1,840*		113	1,840*	Requesting publication of SIR after Examiner action	
	115	110		215	55	Extension for reply within first month	
	116	400		216	200	Extension for reply within second month	
	117	920		217	460	Extension for reply within third month	
	18	1,440		218	720	Extension for reply within fourth month	
	28	1,960		228	980	Extension for reply within fifth month	
	19	320		219	160	Notice of Appeal	
	20	320		220	160	Filing a brief in support of an appeal	
	21	280		221	140	Request for oral hearing	
	38	1,510		138	1,510	Petition to institute a public use proceeding	
	140	110		240	55	Petition to revive – unavoidable	
	141	1,280		241	640	Petition to revive – unintentional	
	142	1,280		242	640	Utility issue fee (or reissue)	
	143	460		243	230	Design issue fee	
	144	620		244	310	Plant issue fee	
	122	130		122	130	Petitions to the Commissioner	
	123	50		123	50	Petitions related to provisional applications	
	126	180		126	180	Submission of Information Disclosure Stmt	
	581	40		581	40	Recording each patent assignment per property (times number of properties)	
	146	740		246	370	Filing a submission after final rejection (37 CFR § 1.129(a))	
	149	740		249	370	For each additional invention to be examined (37 CFR § 1.129(b))	
	179	740		279	370	Request for Continued Examination (RCE)	
	169	900		169	900	Request for expedited examination of a design application	

Other fee (specify) Application for Extension of Patent Term Under 35 U.S.C. 156

1120

The Commissioner is authorized to charge any additional fees to the above noted Deposit Account.

\*Reduced by Basic Filing Fee Paid

SUBTOTAL (3)

(\$1120)

## SUBMITTED BY

Complete (if applicable)

Name (Print/Type)	Kevin Baclan	Registration No. (Attorney/Agent)	34,774	Telephone	415-576-0200
Signature				Date	May 10, 2002

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ATTACHMENT F

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 5,275,813

Issued: January 4, 1994

To: Janet K. Yamamoto et al.

Assignee: The Regents of the University of  
California

For: METHODS AND COMPOSITIONS FOR  
VACCINATING AGAINST FELINE  
IMMUNODEFICIENCY VIRUS

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OFFICE OF PETITIONS

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

**CERTIFICATION**

I hereby certify that this accompanying application for extension of the term of U.S. Patent 5,275,813 under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and two (2) copies thereof.

Respectfully submitted,

Date: May 10, 2002

By: *Kevin L. Bastian*  
Kevin L. Bastian  
Reg. No. 34,774



US005275813A

**United States Patent [19]**

Yamamoto et al.

[11] Patent Number: 5,275,813

[45] Date of Patent: Jan. 4, 1994

## [54] METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

[75] Inventors: Janet K. Yamamoto, Hercules; Niels C. Pedersen, Winters, both of Calif.

[73] Assignee: The Regents of the University of California, Oakland, Calif.

[21] Appl. No.: 739,014

[22] Filed: Jul. 31, 1991

## Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 618,030, Nov. 16, 1990, Pat. No. 5,037,753, which is a continuation of Ser. No. 89,700, Aug. 26, 1987, abandoned.

[51] Int. Cl.<sup>5</sup> ..... A61K 39/12

[52] U.S. Cl. ..... 424/89

[58] Field of Search ..... 424/89; 435/240.2, 235.1

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Primary Examiner—Christine M. Nucker

Assistant Examiner—D. Barnd

Attorney, Agent, or Firm—Townsend and Townsend

Khourie and Crew

## [57] ABSTRACT

Compositions derived from a novel viral isolate designated feline immunodeficiency virus (FIV) include the whole virus, proteins, polypeptides and, polynucleotide sequences derived from the virus; and antibodies to antigenic sites on the virus. These compositions are useful in a variety of techniques for the detection of and vaccination against FIV. Detection methods disclosed include immunoassays for both the virus and antibodies to the virus, and the use of polynucleotide probes to detect the viral genome. Vaccines include both wholly and partially inactivated viruses inactivated cell lines expressing FIV antigens, and subunit vaccines. Whole, live virus is also useful as a model system for predicting the behavior of human immunodeficiency virus (HIV).

3 Claims, 11 Drawing Sheets

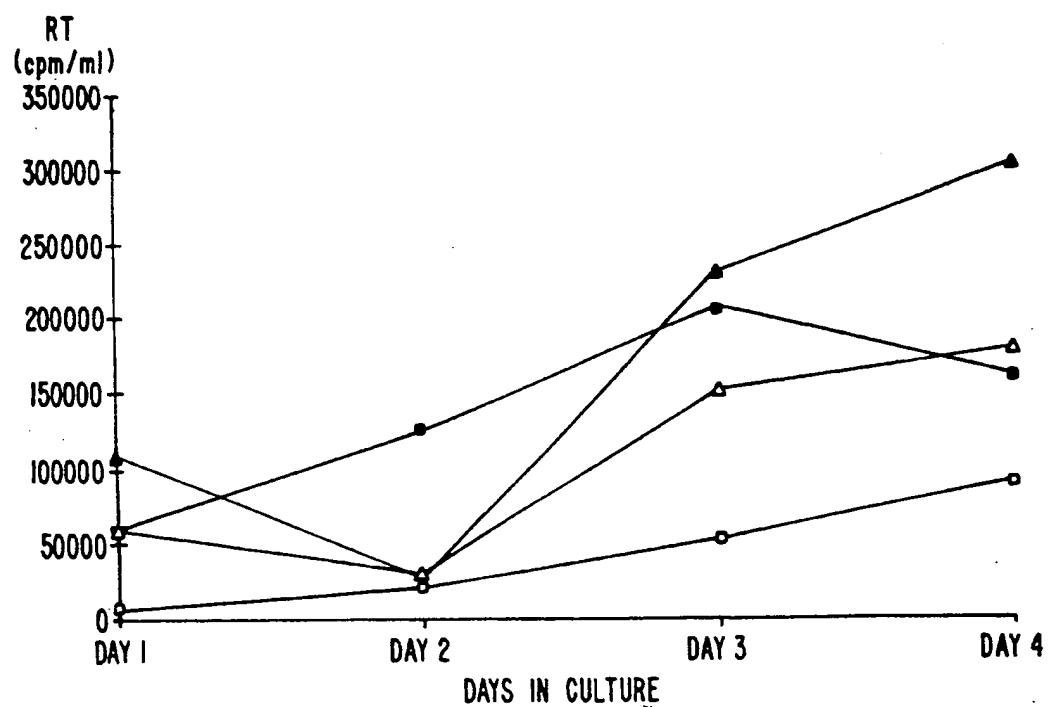
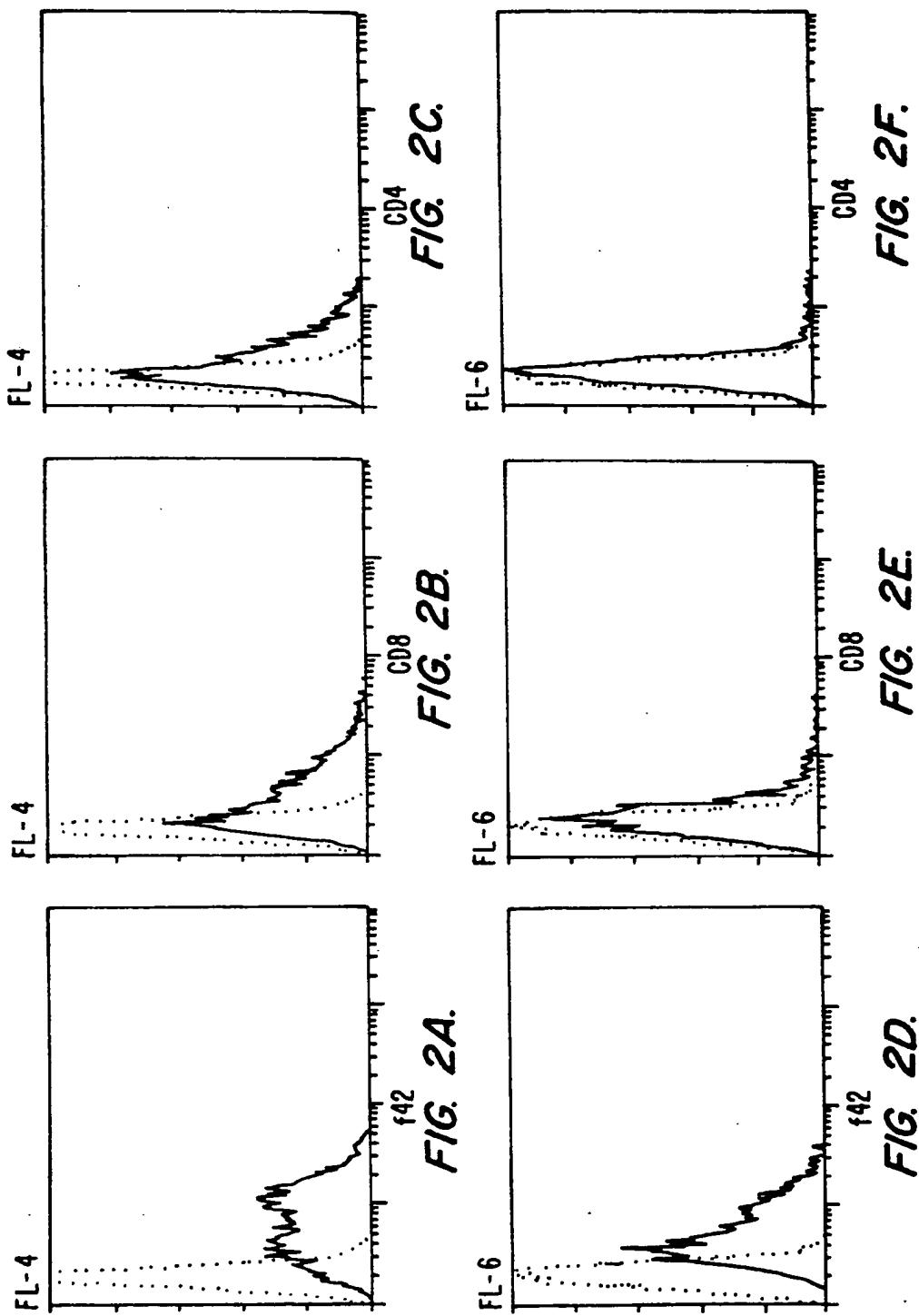
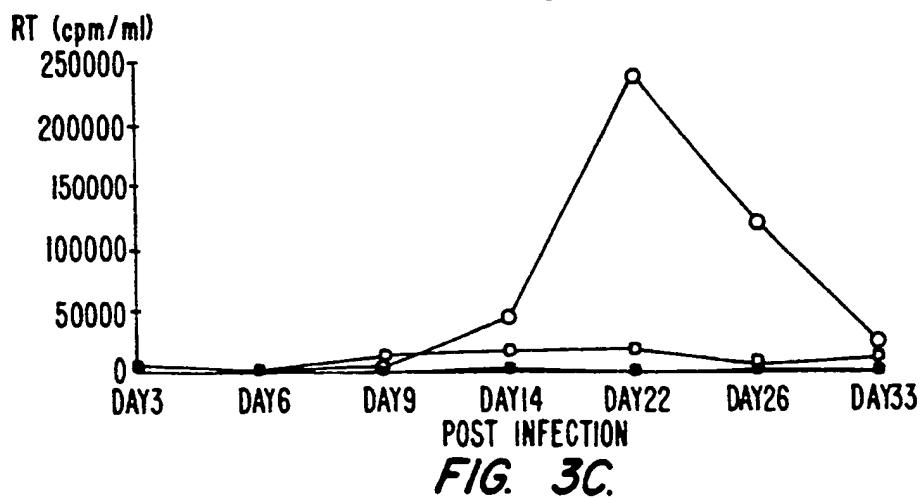
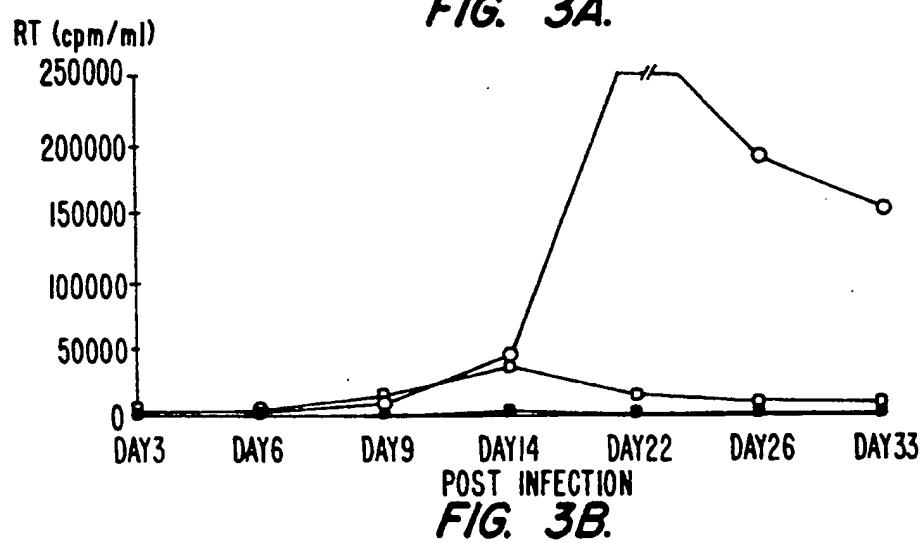
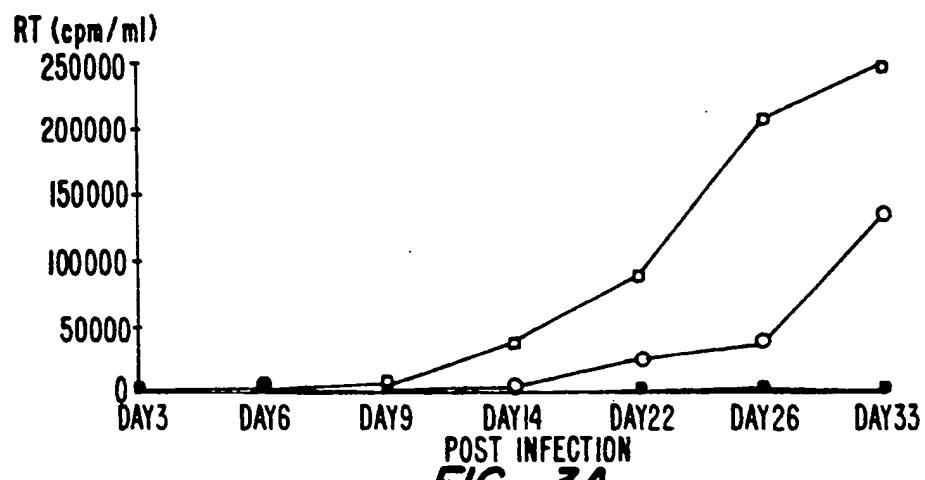
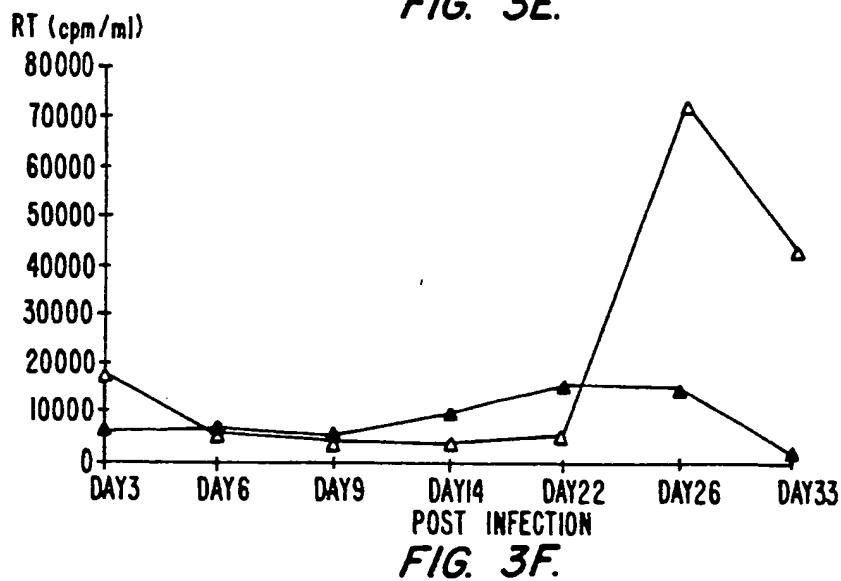
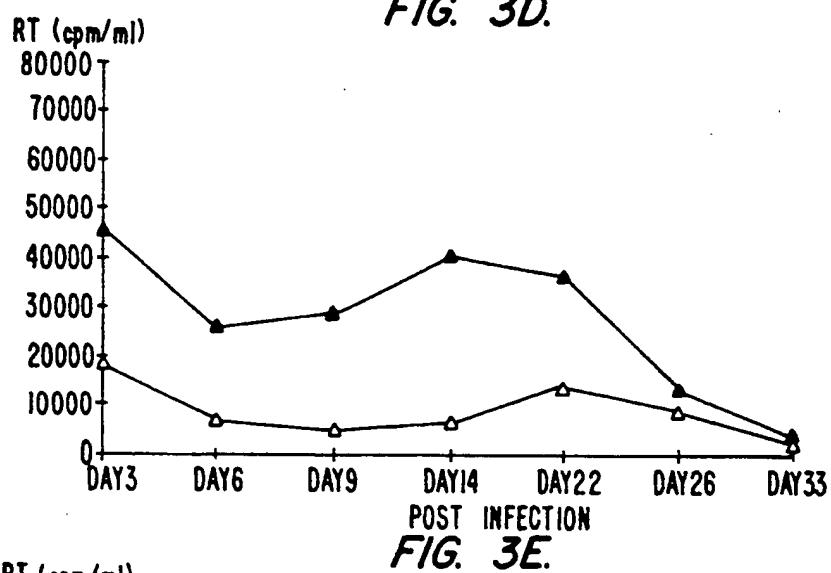
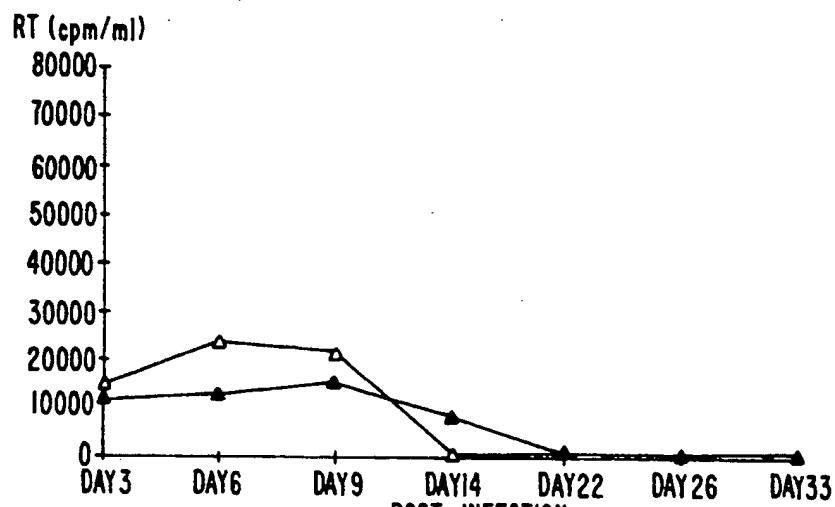
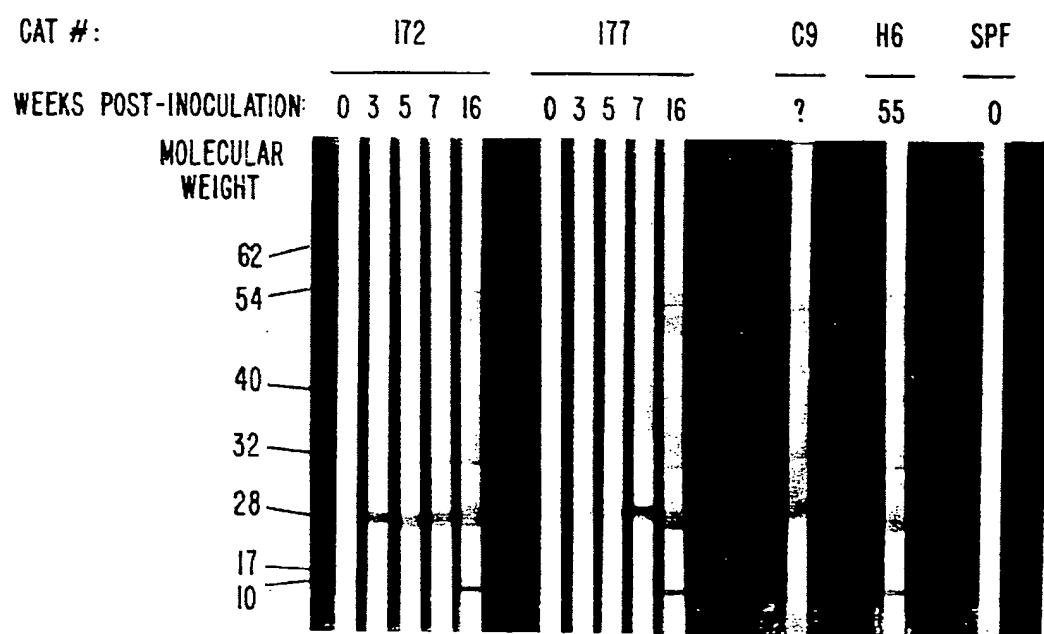


FIG. 1.









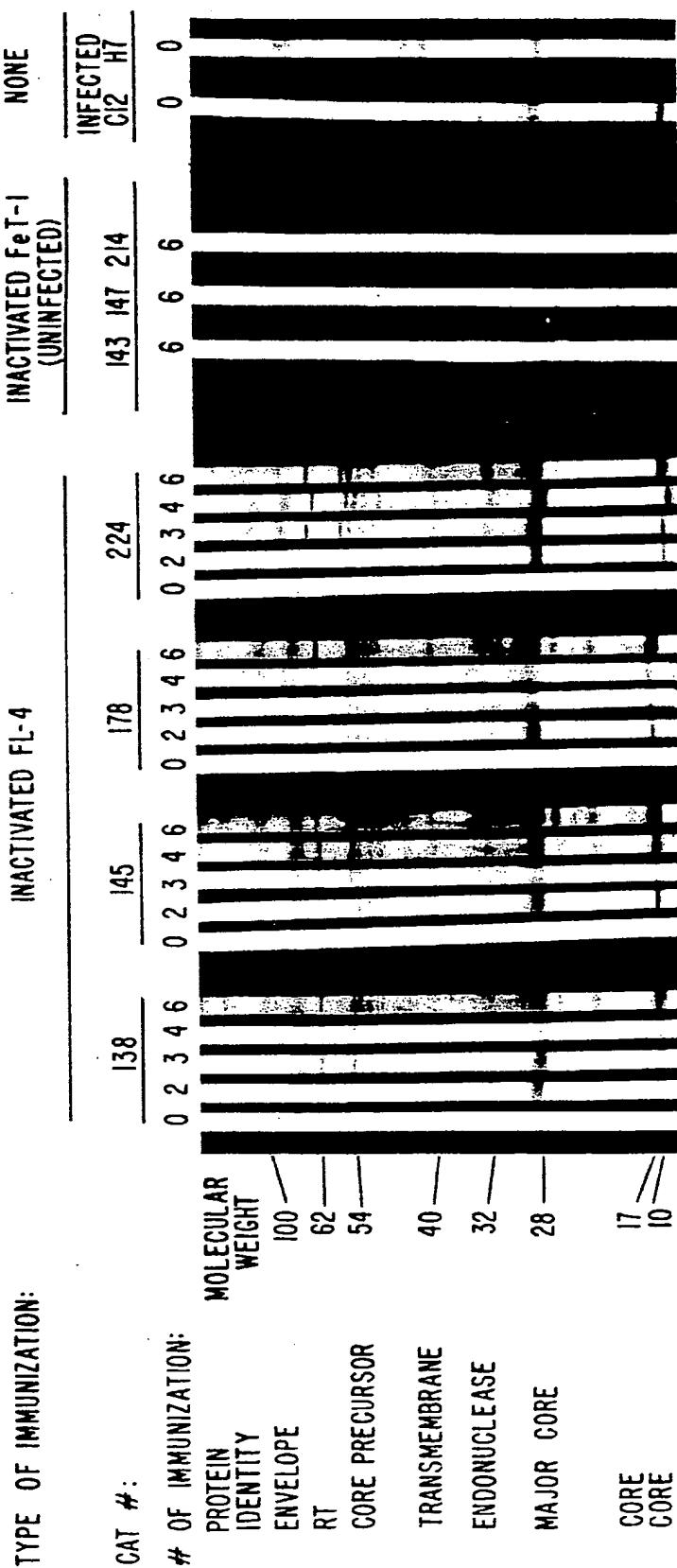


FIG. 5A.

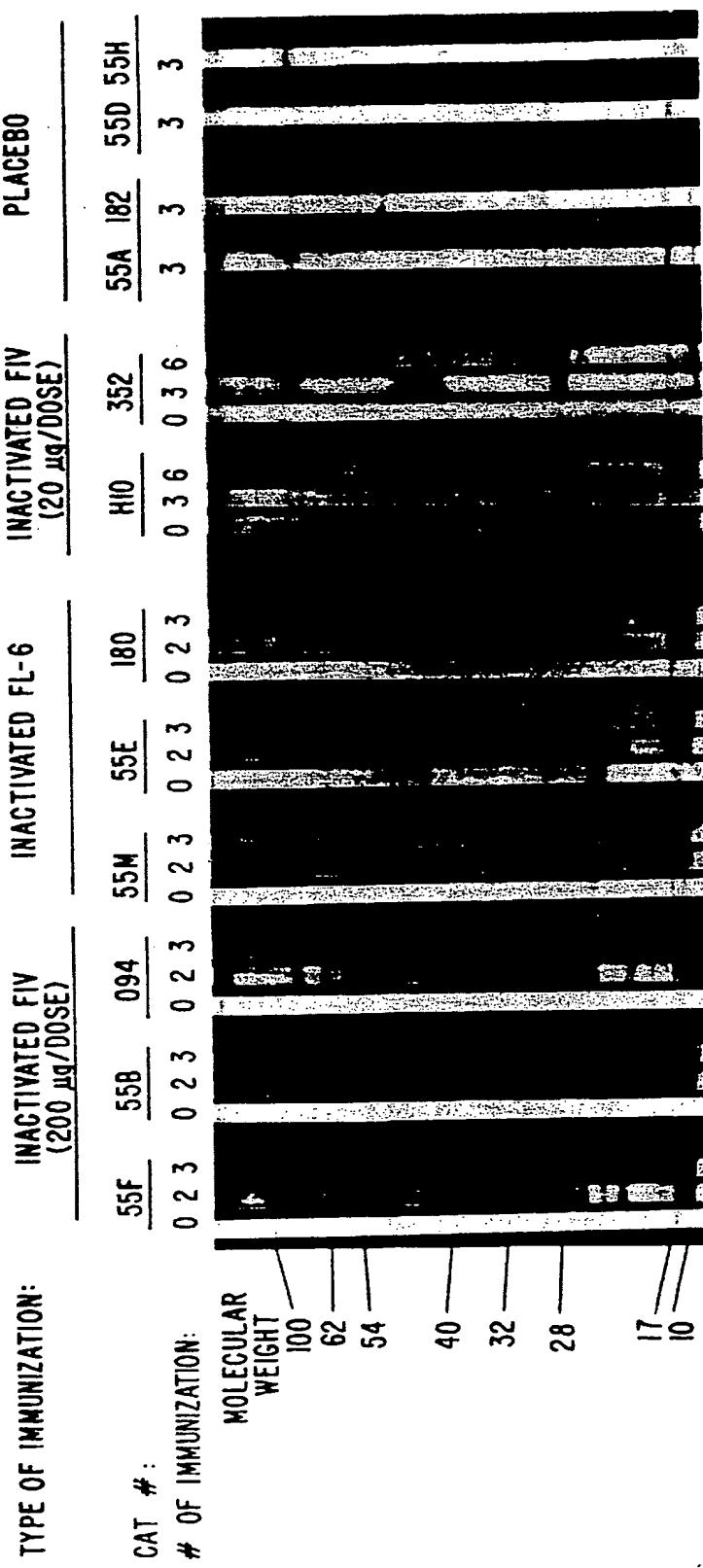
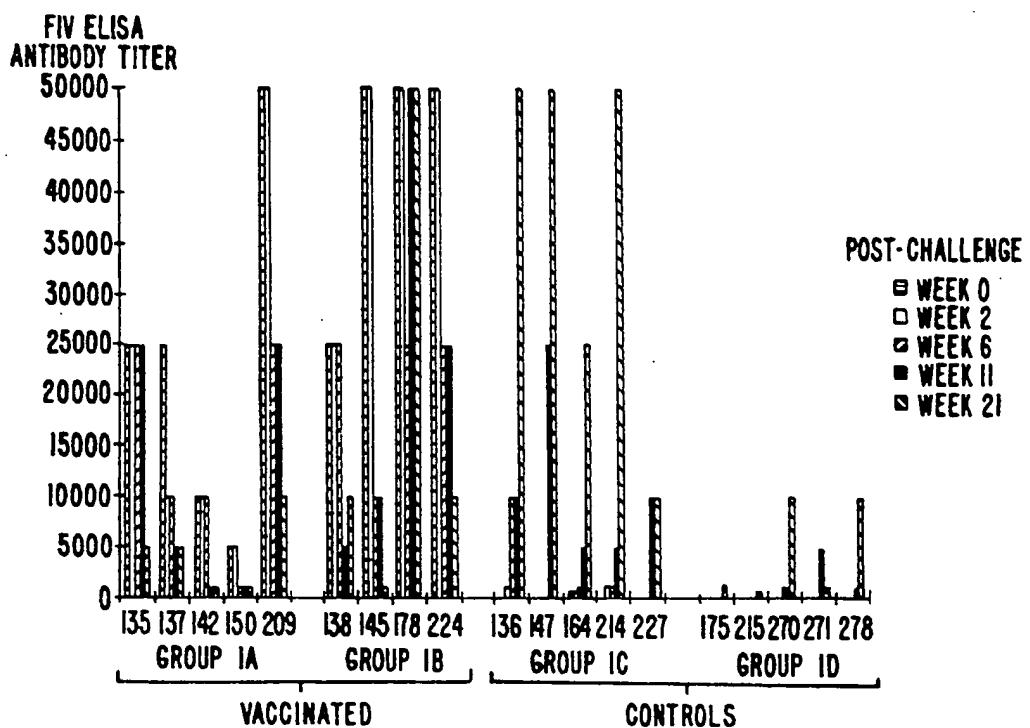
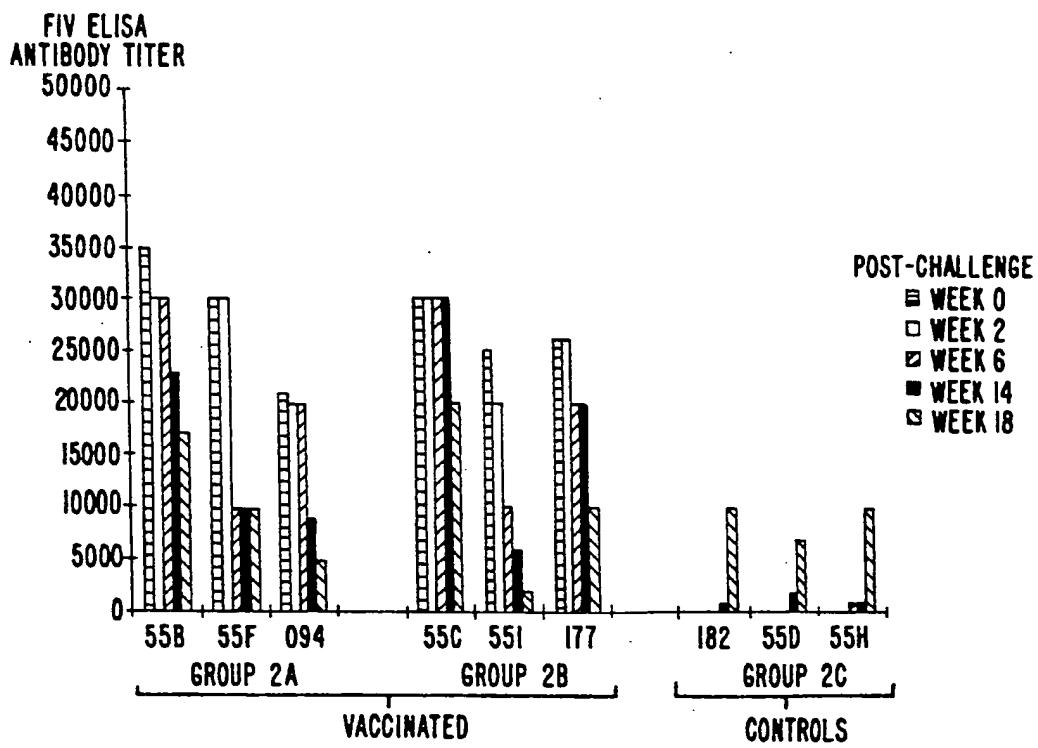


FIG. 5B.



*FIG. 6A.*



*FIG. 6B.*

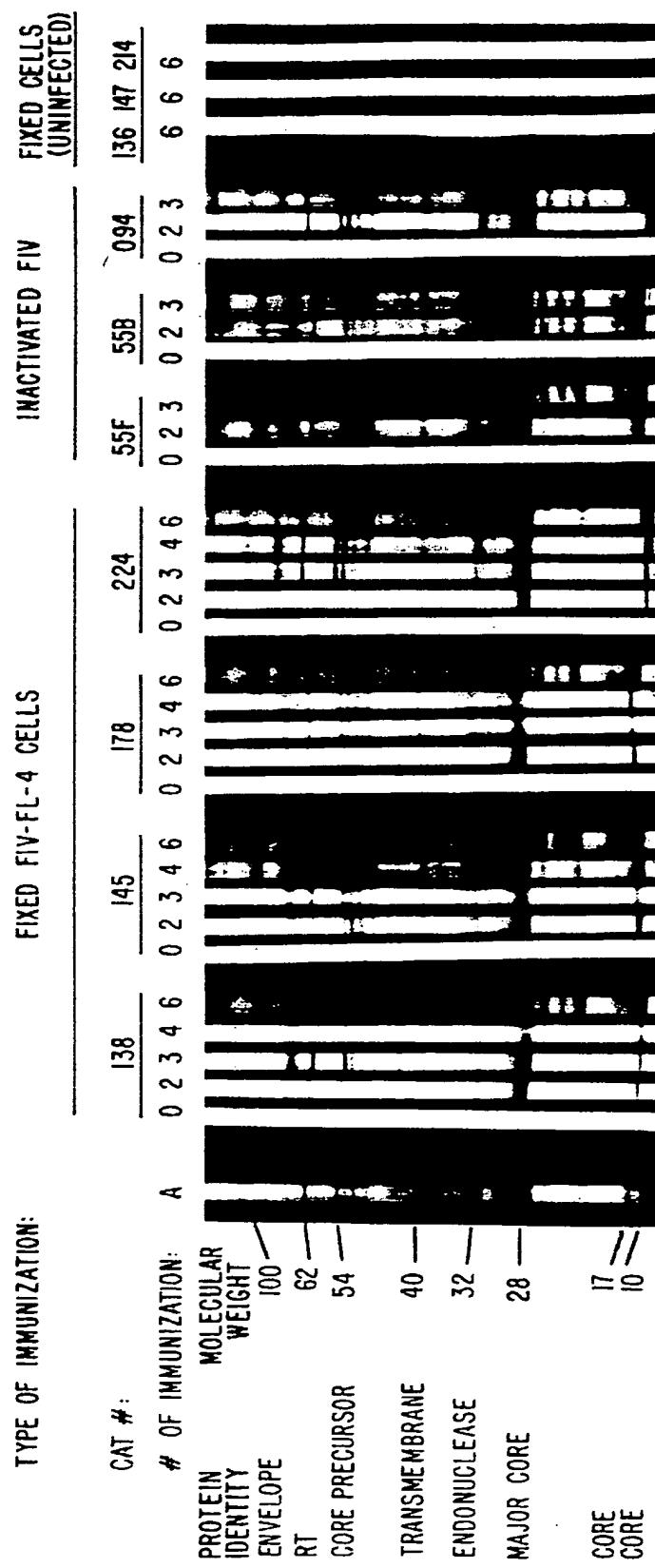


FIG. 7.

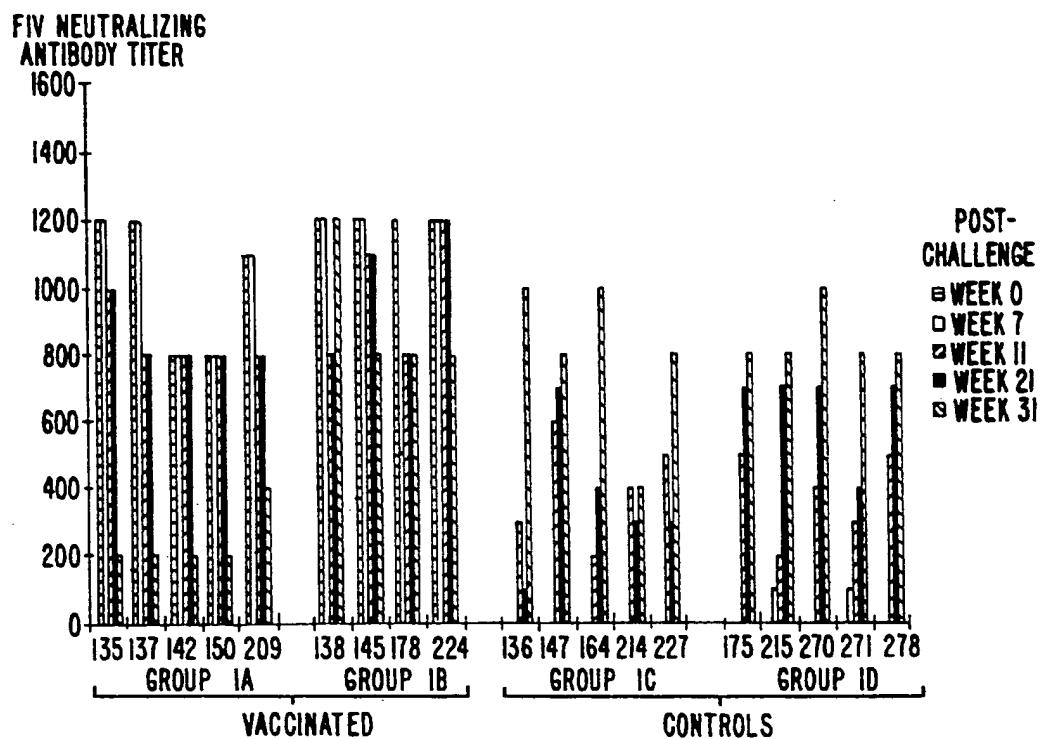


FIG. 8A.

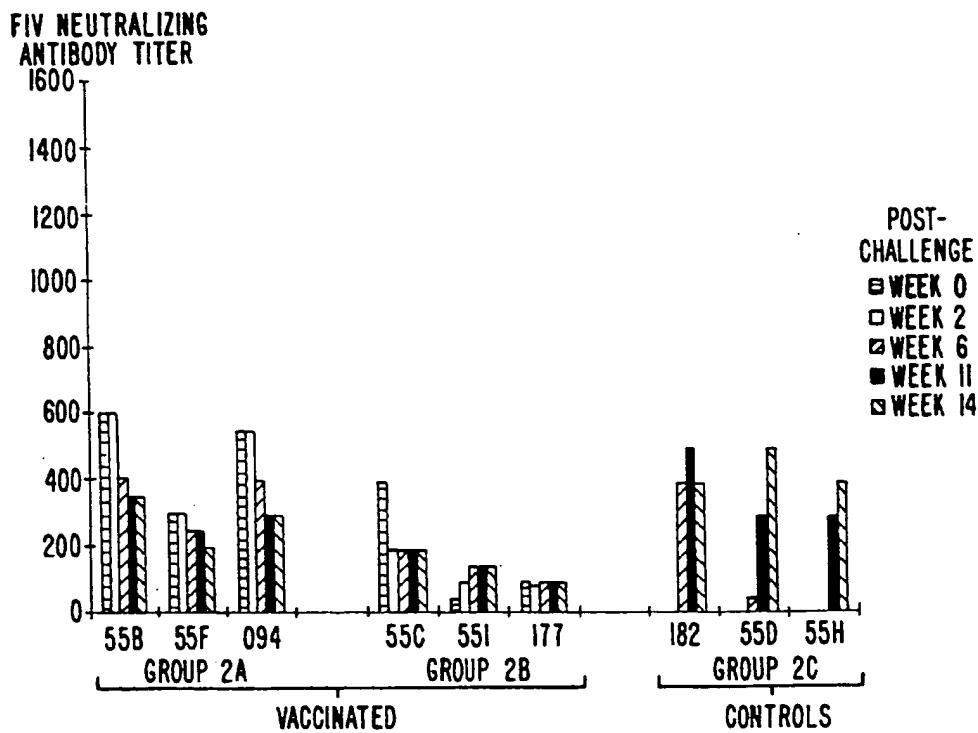


FIG. 8B.

## PROLIFERATION RESPONSE

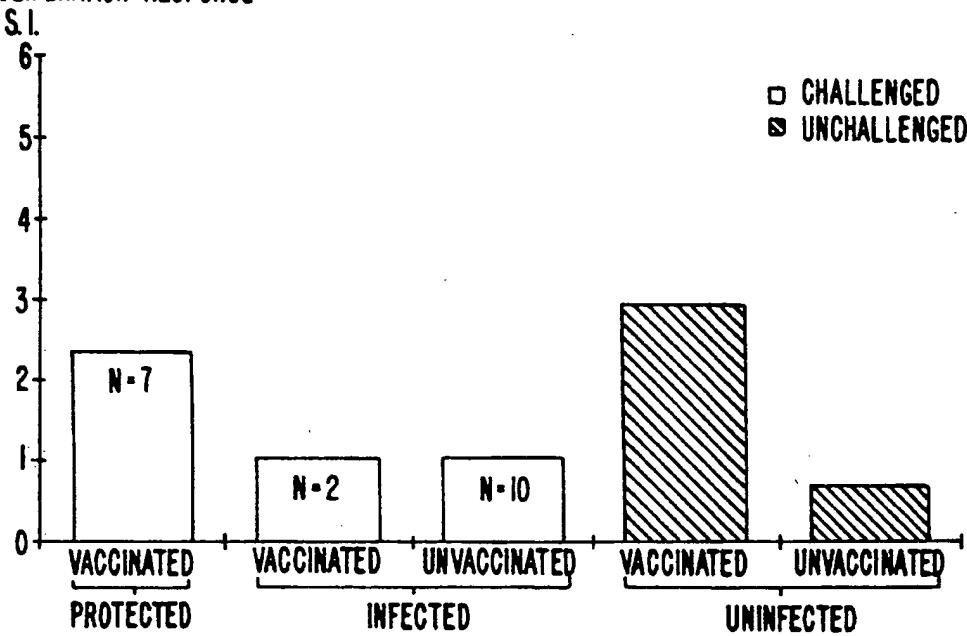


FIG. 9A.

## IL 2 INDUCTION

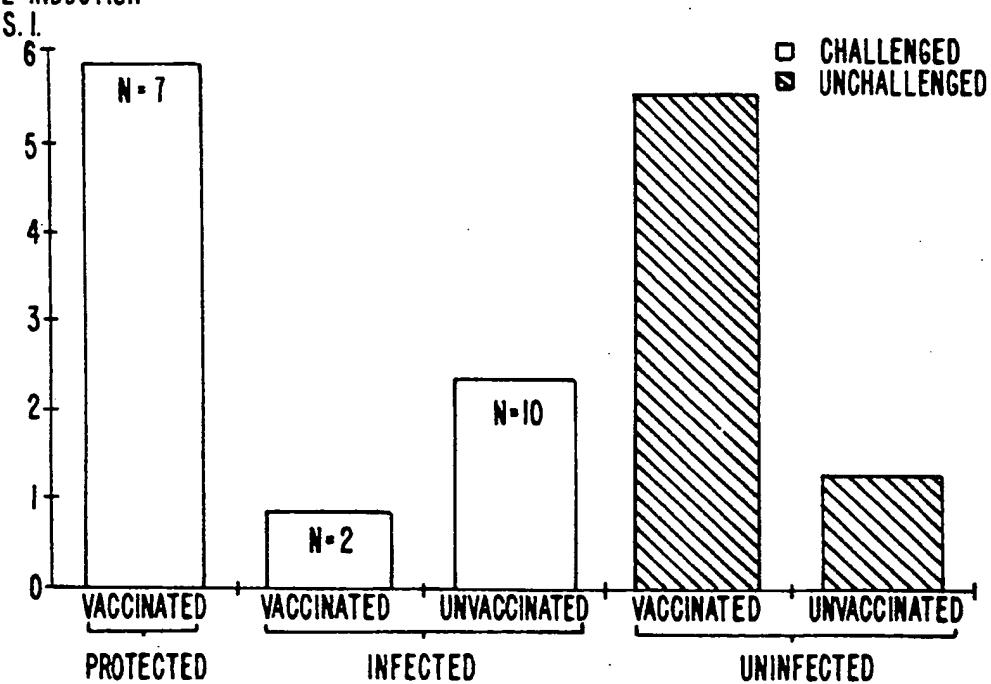


FIG. 9B.

## METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

This invention was made with Government support under Grant No. CA 39016 awarded by the National Institute of Health. The Government has certain rights in this invention.

The present invention is a continuation-in-part of application Ser. No. 07/618,030, filed on Nov. 16, 1990, now U.S. Pat. No. 5,037,753, which was a continuation of application Ser. No. 07/089,700, filed on Aug. 6, 1987, now abandoned. The disclosures of both these applications are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates generally to the detection and treatment of viral infection. More particularly, the invention relates to compositions and methods useful for the diagnosis of and vaccination against infection with a newly-discovered lymphotropic retrovirus, initially designated as feline T-lymphotropic lentivirus and presently designated feline immunodeficiency virus (FIV).

Domestic cats may become infected with several retroviruses, including feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncovirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune-mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

While immunodeficiency syndrome in cats has normally been associated with FeLV, immunodeficiency-like symptoms have been observed in cats which are seronegative for FeLV, usually without alternative explanation. It would be desirable to identify etiological agents other than FeLV which are responsible for causing immunodeficiency in cats. It would be particularly desirable to provide methods and compositions for the detection of and vaccination against such newly-identified etiological agents, and in particular, against FIV.

#### 2. Description of the Background Art

The discovery of feline T-lymphotropic lentivirus (now designated feline immunodeficiency virus) was first reported in Pedersen et al. (1987) Science 235:790-793 Abstracts concerning the discovery of the virus have been presented at the American Association for Cancer Research on May 23, 1987 (Abstract No. 3337); and The Third International Conference on Acquired Immune Deficiency Syndrome, Jun. 1-5, 1987. A poster concerning discovery of the virus was presented at a meeting of the Federation of American Society for Experimental Biology on April 2, 1987.

Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:8088-8092 and 86:4355-4360; and

Talbott et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:5743-5747. Hosie and Jarret (1990) AIDS 4:215-220, describes the serological response of cats infected with FIV.

5 A portion of the experimental data presented in this application was published in AIDS 1990 4 (Suppl. 1):S163-S165.

Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 336:1538-1541; Desrosiers et al. PNAS U.S.A. (1989) 86:6353-6357; Murphey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV 15 gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

### SUMMARY OF THE INVENTION

Compositions and methods are provided for vaccination against a novel feline retrovirus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV). The compositions include vaccines comprising an immunogen capable of eliciting an immune response protective against infection by FIV when administered in an effective amount to a susceptible host. The immunogen will display determinant sites characteristic of the virus, such as those found on the major envelope and core proteins. The preferred immunogens include inactivated whole virus, attenuated whole virus, and inactivated cell lines infected with FIV and which express FIV antigens on their surface. Other immunogens which may find use include polypeptides which mimic the determinant sites, such as FIV peptides (to produce subunit vaccines), antiidiotype antibodies, and the like.

According to the method of the present invention, the vaccine compositions are administered to susceptible hosts, usually cats, in amounts effective to afford immunity against subsequent challenge by FIV. The vaccines may be administered by any conventional route, including subcutaneously, intramuscularly, and oranasally, and will usually be administered at least twice over intervals spaced-apart by one or more weeks to achieve the desired immunity.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: FL-4 ( $\blacktriangle$ ), FL-6 ( $\Delta$ ), FIV-FeT1 ( $\blacksquare$ ), and FIV-CRFK ( $\square$ ) cells were seeded at  $5 \times 10^5$  cells/ml tested daily for the RT activity in their culture fluids. A gradual increase in RT activity was observed over the four days of culture, with peak RT titers detected on Day 4 for all cell cultures except FIV-FeT1 which had it on Day 3 Peak viable cell counts ( $1.0-2.25 \times 10^6$  cells/ml) were observed on Day 3 for all cell cultures except for the FIV-FeT1 culture which had its peak viable cell count ( $1.3 \times 10^6$  cells/ml) on Day 2. The percent cell viabilities during the four days of culturing were 75-90% for FIV-FL-4, 70-90% for FIV-FL-6, 70-80% for FIV-CRFK, and 55-65% for FIV-FeT1.

FIGS. 2A-2F: The FACS profiles of the surface phenotype of FL-4 (FIGS. 2A, 2B, and 2C) and FL-6 (FIGS. 2D, 2E, and 2F) were determined using characterized monoclonal antibodies to feline CD4 (Fel 7), CD8 (FT2), pan T-cell (F42) and the feline light chain and  $\mu$  heavy chain specific (AC5) markers. Both cell lines had cell populations which were positive for CD4 (FIGS. 2C and 2F), CD8 (FIGS. 2B and 2E), and pan T-cell (FIGS. 2A and 2D). Both FL-4 and FL6 cells

tested negative by FACS analysis for surface B cell markers using monoclonal antibodies (AC5) (data not shown). The solid lines represent the FACS profiles of FL-4 and FL-6 cells and the dotted lines represent the FACS profiles of negative control cells. The percentages of FL-4 cells that were positive for CD4, CD8 and pan T-cell markers were 10%, 20%, and 80% respectively. The percentages of FL-6 cells that were positive for CD4, CD8 and pan T-cell markers were <8%, 11%, and 76%, respectively. Depending on the culture conditions, expression of CD4 and CD8 on the cell membrane can be decreased or eliminated. The abscissa represents fluorescence intensity and the ordinate represents relative cell number.

FIGS. 3A-3F: FIV from FL-4 (A,D), FIV-FeT1 (B,E) and FIV-CRFK (C,F) cells were tested for their infectivity on different feline PBLs (A,B,C) and feline thymocytes (D,E,F). Uninfected feline lymphoid cells used in this study were FeT1.1 (■), FeT1.2 (□), FeT1.3 (○), Thy1 (Δ), and Thy2 (▲). All of the FeT1 cells were derived from uninfected PBLs and Thy cells were primary thymocytes obtained from FIV-free kittens. FeT1.1, FeT1.2, and FeT1.3 were subclones of the uninfected FeT1 line, which was the precursor line for FIV-FeT1 cells. The percentage of cells that was positive for CD4 and CD8 markers was <2% and 5% for FeT1.1, <2% and <2% for FeT1.2, and <2% and 4% for FeT1.3, 54% and 4% for Thy 1, and 38% and <2% for Thy2, respectively. Interestingly, FIV from all cell lines were able to either transiently and persistently infect all lymphoid cells except for those from FeT1.2 cultures, whose cells also totally lacked the expression of both CD4 and CD8 markers. Another observation was that FIV from FL-4 and FIV-FeT1 cells infected thymocytes more rapidly but produced a lower titer of virus than those produced by FeT1.1 or FeT1.3 cells. The major difference between the thymocytes and the FeT1.1 or FeT1.3 cells was the large number of CD4+ cells present in the thymocyte cultures. Thus, this observation suggests that the rapid FIV infection of the thymocytes was correlated to the increased number of CD4+ cells.

FIG. 4: Immunoblot analysis was performed on the sera from cats inoculated with 2 ml of cell-free TCF (150,000 cpm/ml RT activity) from either FL-4 (Cat #172) or FL-6 (Cat #177) cultures. The FIV antibody development in these cats was similar to the progression observed previously in SPF cats inoculated with plasma or blood from FIV-infected cats or with TCF from primary PBL cultures (Yamamoto et al., (1988) supra.). Their immunoblot profiles at 16 weeks post-inoculation (pi) resembled those of sera from naturally (Cat #C9) or experimentally (Cat #H6) infected cats. The PBLs from these cats at 10 weeks pi were positive for FIV by virus isolation (data not shown). Both immunoblot and virus isolation results demonstrate that these cats were infected with FIV.

FIGS. 5A and 5B: The immunogenicity of the FIV produced from our FIV-infected cell lines was evaluated in cats. The reactivities of the antibodies produced in cats immunized with either inactivated FL-4 (A) or FL-6 (B) cells or with inactivated FL-4-produced virus (B) were determined by immunoblot analysis. Cats were immunized six-times with inactivated FL-4 or uninfected FeT1 cells and their serum immunoblot profiles were compared to those of serum from cats naturally (Cat #C12) or experimentally (Cat #H7) infected with FIV (A). Cats were also immunized four-times with

inactivated FIV (produced by FL-4 cells) and with inactivated FL-6 cells (B) and evaluated similarly. In the last study, cats were immunized eight-times with 20 µg per dose of inactivated FIV and these results presented. All immunizations were done at two week intervals except for the final interval between the fifth and sixth immunizations in the first study. The adjuvants used were either MDP (A) or a combination of Freund's complete and incomplete adjuvants (B). The predicted molecular masses of the FIV proteins, derived from nucleotide sequence analysis (Olmstead et al. (1989) supra., and Talbott et al. (1989) PNAS U.S.A. 86:5743-5747), have been identified as: 24.5-25.1 kD for major core protein, 14.7-14.9 kD for N-terminal gag protein (minor core protein), 9.5-9.6 kD for nucleocapsid protein (minor core protein), 49.2-49.5 kD for gag precursor protein (core precursor), 61.5 kD for reverse transcriptase (RT), 30.7 kD for endonuclease, 100 kD for major envelope glycoprotein (outer membrane), 36 kD for transmembrane glycoprotein, and 140 kD for precursor envelope glycoprotein. The banding profiles derived from radioimmunoprecipitation analysis using [<sup>3</sup>H]glucosamine (Olmstead et al. (1989) PNAS U.S.A. 86:4355-4360 and Hosie et al. (1990) AIDS 4:215-220) or [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (O'Connor et al. (1989) J. Clin. Micro. 27:474-479), have demonstrated a gp100-120 band for envelope, a gp36-41 diffuse band for transmembrane, and a gp130-140 band for envelope precursor. The molecular weights of the viral protein components, as described by the above analyses, correspond to the immunoblot patterns of 24-28 kD for major core, 15-17 kD for minor core, 10 kD for minor core, 54-55 kD for core precursor, 62 kD for RT, 32 kD for endonuclease, 37-44 kD (diffuse band) for transmembrane, and 100-120 kD for envelope (Yamamoto et al (1988) supra.; Hosie et al. (1990) supra.; and O'Connor et al. (1989) J. Clin. Micro. 27:474-479). In this study, the development of antibodies to major core protein p28 was observed prior to the development of antibodies to the envelope glycoprotein gp100 in both immunization studies (A and B). Our immunoblot analysis of the sera from immunized cats closely resembled the immunoblot profiles of FIV-infected cats previously published by our laboratories and others (Yamamoto et al. (1988) supra.; Hosie et al. (1990) supra.; and O'Connor et al. (1989) J. Clin. Micro. 27:474-479). Comparison of high dose (200 g) indicates that large amounts of viral proteins are required to adequately and rapidly induce FIV antibodies (B). The immunoblot numbers represent the cat identification numbers.

FIGS. 6A and 6B: The FIV IgG antibody titer was measured by enzyme-linked immunosorbent assay (ELISA) using 250 ng/microwell of sucrose-gradient purified FIV as substrate and biotinylated goat anti-cat IgG (Vector Laboratories, BA-9000) as conjugating antibody (Pedersen et al (1987) Science 235:790-793). Sera from the different bleeding dates of each cat were serially diluted and assayed simultaneously in a single test. The results are based on two separate ELISA testings. Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIG. 7: Immunoblot analysis was performed on sera at a final dilution of 1:50 from cats immunized with fixed cell-virus or inactivated whole-virus vaccines. Results presented are those from cats immunized with fixed FIV-FL-4 cells (Group 1B), inactivated FIV

(Group 2A), or fixed uninfected FeT1 cells (Group 1C). Lane A is an immunoblot profile of a SPF cat experimentally infected with FIV.

FIGS. 8A and 8B: The neutralizing antibody titers to FIV were assayed the FIV-susceptible feline lymphoid cell line FeT1. In brief, diluted samples of heat-inactivated serum (56° C. for 30 min) were incubated with 100 tissue culture infective doses (TCID<sub>50</sub>) of FIV (Petaluma strain) for 45 min at 37° C. in a 25-cm flask. The FeT1 cells were added to this mixture at a final concentration of  $2 \times 10^5$  cells/ml. After three days of culturing, the cells were washed once with Hank's balanced salt solution to remove residual virus from the culture and then resuspended in fresh culture media (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 10 mM HEPES buffer, 50 µg/ml gentamicin, 1 × 10<sup>-5</sup>M 2-mercaptoethanol, and 100 U/ml human recombinant IL-2). Virus infection was monitored by Mg<sup>++</sup>-dependent RT assays of the culture fluid. The serum was considered positive for neutralizing antibodies when RT activity was  $\leq 50\%$  of the infected control culture which had no serum exposure. Nonspecific antiviral activity (i.e., interferon activity) was not detected in the heat-inactivated serum samples using the antiviral assay with vesicular stomatitis virus (Yamamoto et al. (1986) *Vet. Immunol. Immunopathol.* 11:1-19). Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIGS. 9A and 9B: Cellular immunity of the cats immunized with the fixed cell-virus vaccine was monitored by assaying the PBLs for their ability to proliferate (FIG. 9A) or produce IL-2 (FIG. 9B) upon stimulation with inactivated whole FIV (1.25% paraformaldehyde inactivated). The PBLs were isolated from blood harvested at 27 weeks pc from all challenged animals and at 14 weeks post-immunization from unchallenged cats. The proliferation assay consisted of <sup>3</sup>H-thymidine incorporation by PBLs ( $1 \times 10^5$  cells/microwell) upon stimulation with inactivated FIV (4.5 µm/microwell) for five days at 37° C. Similarly, 1.5 ml-cultures of PBL ( $1 \times 10^6$  cells/ml) were incubated with FIV antigens (50 µm/ml) for two days and the culture fluid was assayed for IL-2 titer. The IL-2 assay consisted of measuring the amount of <sup>3</sup>H-thymidine incorporation of the IL-2-dependent murine HT-2C cells in presence or absence of IL-2 containing samples (Gillis et al. (1978) *J. Immunol.* 120:2027-2032). When compared to infected, unvaccinated control cats, the vaccine protected cats and the vaccinated but unchallenged cats responded significantly (stimulation index  $\geq 2.0$ ) to FIV antigenic stimulation in both proliferation ( $P < 0.001$ ) and IL-2 induction ( $P < 0.001$ ) assays. The P value was derived by using two-tailed t-test. Part A presents the results from the proliferation assay and part B the results from the IL-2 induction assay.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A novel virus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV) has been discovered and isolated in substantially pure form. The virus is infectious in cats, causing a wide variety of symptoms, including abortion, alopecia, anemia, chronic rhinitis, conjunctivitis, diarrhea, emaciation, enteritis, gingivitis, hematochezia, neurologic abnormalities, periodontitis, and seb-

orrheic dermatitis. The course of the disease is usually fatal.

The etiology, pathogenesis, and morphology of FIV closely resemble those of human immunodeficiency virus (HIV) and simian T-lymphotropic virus III (SAIDS), which cause acquired immunodeficiency syndrome in humans and primates, respectively. FIV does not appear to be antigenically related to HIV or to SAIDS, but rather appears to be a species-adapted lentivirus that has existed in cats for some time. Preliminary surveys conducted by the inventors herein indicate that FIV infection in cats may be widespread, possibly accounting for a significant proportion of the immunodeficiency symptoms found in cats who are free from FIV infection.

FIV is a feline immunodeficiency virus characterized as a retrovirus, more specifically as a lentivirus, which is tropic for T-lymphocytes of the host which it infects. The virus is also characterized by horizontal transmission, and may further be characterized by vertical transmission in at least some cases.

It is expected that FIV is polymorphic, and reference to FIV in the present application is intended to encompass the entire FIV family, including a variety of strains which share substantial amino acid sequence and nucleotide sequence homology and which are immunologically related. Substantial amino acid sequence homology means at least about 75% homology, usually at least about 80% homology, and frequently 90% homology and above in at least some of the viral genes and proteins. For example, the env, gag, or pol regions may display the requisite homology, while the genome as a whole does not. In such cases, so long as the viruses are immunologically related, the viruses will be considered to be FIV within the ambit of the present invention.

By immunologically related it is meant that the various strains will display substantial serologic cross-reactivity with the newly-discovered strain which has been deposited. Serologic cross-reactivity is defined as the ability of an antiserum or antibodies specific for the deposited FIV strain to react with other FIV strains as well as the deposited strain. Usually, immunologically related strains will cross-react with antibodies specific for more than one epitopic site, usually more than five epitopic sites, and frequently ten or more epitopic sites.

Conveniently, FIV strains may be identified by Western blot analysis where purified virus is disrupted with a suitable detergent, e.g., sodium dodecyl sulfate, and separated on a slab gel by electrophoresis. The separated polypeptide bands are transferred from the gel to nitrocellulose filter paper and visualized with labelled antibody. The molecular weights of the various resolved bands may then be determined by comparison to known molecular weight standards. Substantial similarity between the Western blot analysis of an unidentified virus and that of a known FIV virus indicates that the unknown virus is likely an FIV virus.

Other FIV isolates have been characterized, indicating that the nucleotide sequence of the envelope gene varies by no more than about 15% among isolates. Such isolates, from different regions, are described in Masashi et al. (1990) In: Proc. 6th Intnl. Conf. AIDS, June 20-24, San Francisco, Abstract Th.A. 284 (Japanese isolate); Phillips et al. (1990) *J. Virol.* 64:4605-4613 (San Diego, California); Olmsted et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:2448-2452 (Petaluma, California); Talbot et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5743-5747 (Petaluma, California); Rigby et al. (1991)

In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, Cameron House, Loch Lomond, Scotland, May 28-31, page 42 (Scotland); and Siebelink et al. (1991) In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, supra. (The Netherlands). Any of these isolates could be used for preparing vaccines and cell lines according to the present invention.

FIV encodes an RNA-dependent DNA polymerase (reverse transcriptase) which is Mg.<sup>+2</sup>-dependent with maximal activity occurring at a Mg.<sup>+2</sup> concentration of approximately 5 mM and pH of approximately 7.8. FIV bands at a density of about 1.15 g/cm<sup>3</sup> in a continuous sucrose gradient. Western blotting of FIV-infected cell lysate yields major bands at approximately 22 to 28 kD, usually about 26 kD; 50 to 60 kD, usually about 55 kD; and 28 to 36 kD, usually about 32 kD.

FIV may be isolated from the sera of infected cats by conventional techniques. For example, peripheral blood lymphocytes (PBL) may be isolated from the blood of infected cats and placed in suitable culture media. The cultures are incubated, with normal PBL's being periodically introduced to the culture in order to maintain its viability as the original cells are killed by the virus. The infected cells should be placed in fresh culture medium periodically, and the virus may be recovered from the supernatant of the cell culture by sucrose-gradient separation, or other known separation techniques.

The FIV may also be obtained from other specimens, particularly from the lymph tissues of infected animals. The lymph tissues are broken and then suspended in culture medium, and the procedures described above are then carried out.

Compositions according to the present invention include the whole virus, as well as portions of the virus. The whole virus may be maintained in vitro culture, as described above, or may be viably frozen at a temperature at or below about -78° C. (solid CO<sub>2</sub>-dry ice), usually in the presence of agents which promote amorphous, vitreous solidification rather than crystallization. Suitable agents include glycerol and dimethylsulfoxide. Portions of the FIV of particular interest include the structural and regulatory proteins encoded by the FIV genome, including the envelope and core proteins, and fragments thereof.

The FIV may also be maintained in chronically infected cell lines, particularly T-cell lines, as described in detail in the Experimental section hereinafter. For example, interleukin 2 (IL-2)-dependent T-cell lines can be infected with FIV and maintained in IL-2-supplemented culture media. IL-2-independent cell lines can then be prepared by repeated subculturing with a gradual depletion of IL-2. Surviving cultures can then be maintained in culture free from IL-2. The IL-2-independent FIV-infected cell lines have been found to possess enhanced viability and a reduced percentage of syncytial cells when compared to IL-2-dependent FIV-infected cell lines. See, Experimental section hereinafter.

The FIV used for infecting the cell lines may be isolated from infected cats, as described above, or may be obtained from the deposited Petaluma strain of the virus (A.T.C.C. VR 2186).

Particular non-infected feline T-lymphocyte cell lines (IL-2 dependent) which may be infected to produce chronically FIV-infected cell lines are designated FeT-1M (A.T.C.C. Accession No. CRL 10775) and FeT-2D

(A.T.C.C. Accession No. CRL 10774), both deposited at the American Type Culture Collection, Rockville, Maryland, on Jun. 7, 1991.

Particular FIV-infected cell lines (IL-2 independent) which have been established from FeT-1M are FL-4 (A.T.C.C. Accession No. CRL 10772) and FL-6 (A.T.C.C. Accession No. CRL 10773), both deposited at the American Type Culture Collection on Jun. 7, 1991. Both these cell lines have been found to be prolific producers of FIV.

FeT-1M, FeT-2D, FL-4 and FL-6 were developed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

Polypeptides of the present invention will be either haptenic or antigenic, including at least six amino acids, usually at least nine amino acids, and more usually twelve or more amino acids found contiguously within one of the natural FIV proteins. Polypeptides will generally correspond to at least one epitopic site which is characteristic of FIV. By characteristic, it is meant that the epitopic site will allow immunologic detection of the virus in a physiological sample with reasonable assurance. Usually, it will be desirable that the epitopic site be immunologically distinct from (i.e., not cross-reactive with antibodies which recognize) viruses other than FIV. In some cases, however, it may be desirable that the epitopic site be immunologically similar to other viruses.

The FIV polypeptides may be natural, i.e., including the entire FIV protein or fragments thereof isolated from a natural source, or may be synthetic. The natural polypeptides may be isolated from the whole virus which is obtained as described above by conventional techniques, such as affinity chromatography. Conveniently, polyclonal or monoclonal antibodies obtained according to the present invention (as described in more detail hereinbelow) may be used to prepare a suitable affinity column by well-known techniques. Such techniques are taught, for example, in Hudson and Hay, *Practical Immunology*, Blackwell Scientific Publications, Oxford, United Kingdom, 1980, Chapter 8.

Synthetic polypeptides which are immunologically cross-reactive with a natural FIV protein may be produced by either of two general approaches. First, polypeptides having fewer than about 100 amino acids, more usually fewer than about 80 amino acids, and typically fewer than about 50 amino acids, may be synthesized by the well-known Merrifield solid-phase synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc., 85:2149-2156).

The second and preferred method for synthesizing the polypeptides of the present invention involves the expression in cultured cells of recombinant DNA molecules encoding a desired portion of the FIV genome. The portion of the FIV genome may itself be natural or synthetic, with natural genes obtainable from the isolated virus by conventional techniques. Of course, the genome of FIV is RNA, and it will be necessary to transcribe the natural RNA into DNA by conventional techniques employing reverse transcriptase. Alternatively, polynucleotides may be synthesized by well-known techniques. For example, short single-stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981), Tett. Letters 22:1859-1862. Double-stranded fragments may then be obtained either by synthesizing the complementary strand and then annealing the

strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The natural or synthetic DNA fragments coding for the desired FIV protein or fragment may be incorporated in a DNA construct capable of introduction to and expression in *n* *vitro* cell culture. Usually, the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria. They may also be intended for introduction and integration within the genome of cultured mammalian or other eukaryotic cells. DNA constructs prepared for introduction into bacteria or yeast will include a replication system recognized by the host, the FIV DNA fragment encoding the desired polypeptide product, transcriptional and translational initiation regulatory sequences joined to the 5'-end of the FIV DNA fragment, and transcriptional and translational termination regulatory sequences joined to the 3'-end of the fragment. The transcriptional regulatory sequences will include a heterologous promoter which is recognized by the host. Conveniently, a variety of suitable expression vectors are commercially available for a number of hosts.

To be useful in the detection methods of the present invention, the polypeptides are obtained in a substantially pure form, that is, typically from about 50% W/W or more purity, substantially free of interfering proteins and contaminants. Preferably, the FIV polypeptides are isolated or synthesized in a purity of at least 80% W/W, and more preferably, in at least about 95% W/W purity. Using conventional protein purification techniques, homogeneous polypeptide compositions of at least about 99% W/W purity can be obtained. For example, the proteins may be purified by use of the antibodies described hereinafter using the immunoabsorbant affinity columns described hereinabove.

Once a sufficient quantity of natural or synthetic FIV polypeptides have been obtained, polyclonal antibodies specific for FIV may be produced by *n* *vitro* or in vivo techniques. In vitro techniques involved in vitro exposure lymphocytes to the antigenic polypeptides, while in vivo techniques require the injection of the polypeptides into a wide variety of vertebrates. Suitable vertebrates are non-human, including mice, rats, rabbits, sheep, goats, and the like. Polypeptides having more than about thirty amino acids, usually more than about fifty amino acids, may serve directly as the immunogen. If the polypeptide is smaller than about 10kD, particularly less than about 6kD, however, it may be necessary to join the polypeptide to a larger molecule to elicit the desired immune response. The immunogens are then injected into the animal according to a predetermined schedule, and the animals are bled periodically with successive bleeds having improved titer and specificity. Injections may be made intramuscularly, subcutaneously, or the like, and an adjuvant, such as a combination of complete and incomplete Freund's adjuvant, will usually be employed. The whole virus can also be used as the immunogen, although selection of antibodies specific for a particular determinant will be more difficult.

If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having the desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse, is hyperimmunized with the desired antigen by the method just described. The vertebrate is then killed,

usually several days after the final immunization, the spleen removed, and the spleen cells immortalized. The manner of immortalization is not critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1976) Eur. J. Immunol. 6:511-519. Other techniques include EBV transformation, transformation with oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with a fusion partner, the manner of fusion is not critical and various techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of a non-ionic detergent, usually polyethylene glycol, and other additives such as Dulbecco's Modified Eagle's medium, for a few minutes. At the end of the fusion, the non-ionic detergent is rapidly removed by washing the cells. The fused cells are promptly dispensed in small culture wells (usually in a microtiter plate at relatively low density, ranging from about one to  $5 \times 10^5$  cells/well), in a selective medium chosen to support growth of the hybrid cells while being lethal to the myeloma cells. Usually, the myeloma cell line has been mutated to be sensitive to a lethal agent, typically being HAT sensitive, and the medium includes a HAT concentration sufficient to inhibit the proliferation of the unfused myeloma cells.

After sufficient time, usually from about one to two weeks, colonies of hybrids are observed and plates containing hyperpositive wells are identified. The plates and wells having only one colony per well are selected, and supernatants from these wells are tested for binding activity against FIV or a particular FIV protein. Once positive hybridomas are identified, the cell line can be maintained as a viable culture and/or a quantity of the virus may be grown out, separated, and stored by lyophilization.

Depending on the desired use for the antibodies, further screening of the hybridomas may be desirable. For use in immunodiagnostic assays, antibodies having very high specificity and affinity for the antigenic site are desirable.

Once the desired hybridomas have been selected, monoclonal antibodies may be isolated from supernatants of the growing colonies. The yield of antibodies obtained however, is usually low. The yield may be enhanced by various techniques, such as injection of the hybridoma cell line into the peritoneal cavity of a vertebrate host. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Proteinaceous and other contaminants will usually be removed from the monoclonal antibodies prior to use by conventional techniques, e.g., chromatography, gel filtration, precipitation, extraction, or the like.

The polypeptides and antibodies of the present invention may be used with or without modification for the detection of or vaccination against FIV infection. Frequently, the polypeptides and antibodies will be labelled by joining, either covalently or non-covalently, a substance which provides for detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Some of the labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescers, chemiluminescers, magnetic particles and the like. Patents teaching the use of such labels include U.S.

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Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies and polypeptides prepared as described above can be used in various immunological techniques for detecting FIV and anti-FIV antibodies in physiological specimens, particularly body fluid samples, including blood, plasma, serum, urine, and the like, and cell samples, such as lymphocytes. Depending on the nature of the sample, both immunoassays and immunohistochemical staining techniques may find use.

Liquid phase immunoassays and Western blot analysis will find use in detection of FIV in body fluids, particularly blood and urine. The use of antibodies in protein binding assays is well established. Numerous competitive and noncompetitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available. Detailed methods for detecting the presence of the viruses in serum samples are set forth in the Experimental section hereinafter. Additionally, enzyme linked immunosorbent assays (ELISA) for detecting presence of antibodies to FIV in blood are also set forth in the Experimental section.

Compositions of the present invention are also useful in preparing vaccines for protection against FIV infection. For example, the whole virus and/or FIV-infected cell lines may be wholly or partially inactivated and utilized as an immunogen in a vaccine composition. Partial inactivation may be achieved by passage at elevated temperatures or by contact with mutagens, such as ultraviolet light, ethyl methanesulfonate, and the like. Complete inactivation may be achieved by contact with other agents, including formalin, paraformaldehyde, phenol,  $\alpha$ -lactopropionate, ultraviolet light, heat, psorlens, platinum complexes, ozone and other viricidal agents.

Specific methods for the preparation of inactivated whole virus and FIV-infected cell line vaccines are described in detail in the Experimental section hereinafter. Conveniently, the source of whole FIV can be FIV-infected cell lines which have been found to be prolific producers, such as FL-4 and FL-6. Inactivated FL-4 and FL-6 are also suitable for preparing inactivated or attenuated whole cell vaccines.

The viral proteins and portions thereof, prepared as described above, may also be used in the preparation of subunit vaccines prepared by known techniques. Polypeptides displaying antigenic regions capable of eliciting protective immune response are selected and incorporated in an appropriate carrier. Alternatively, an antigenic portion of a viral protein or proteins may be incorporated into a larger protein by expression of fused proteins. The preparation of subunit vaccines for other viruses is described in various references, including Lerner et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:3403 and Bhatanagar et al. (1982) proc. Natl. Acad. Sci. U.S.A. 79:4400. See also, U.S. Pat. Nos. 4,565,697 (where a naturally-derived viral protein is incorporated into a vaccine composition); 4,528,217 and 4,575,495 (where synthetic peptides forming a portion of a viral protein are incorporated into a vaccine composition). Other methods for forming vaccines employing only a portion of the viral proteins are described in U.S. Pat. Nos. 4,552,757; 4,552,758; and 4,593,002. The relevant portions of each of these cited references and patents are incorporated herein by reference.

The vaccines prepared as described above may be administered in any conventional manner, including

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oronasally, subcutaneously, intraperitoneally or intramuscularly, except that oronasal administration will usually not be employed with a partially inactivated virus vaccine. Adjuvants will also find use with subcutaneous and intramuscular injection of completely inactivated vaccines to enhance the immune response. The preparation of viral vaccine compositions optionally employing adjuvants is described in numerous standard references, such as *Remington's Pharmaceutical Sciences*, 10 Mack Publishing Co., Easton, Pa., 16th ed., 1982, the disclosure of which is incorporated herein by reference.

The dosage form and immunogen content of the vaccine will vary depending on the nature of the immunogen (i.e., whole virus, infected cell, or subunit) and the route of administration. Usually, a single dose will have a total volume including carrier, adjuvant, and any other components, in the range from about 0.1 ml to about 5 ml, more usually being from about 0.5 ml, more usually being from about 0.5 ml to about 3 ml. The amount of inactivated or attenuated whole FIV in each dose will usually be in the range from about 0.1 mg to about 5 mg, usually being from about 0.2 mg to 2 mg. For inactivated FIV-infected cell lines, each dose will typically contain from about  $10^6$  to  $10^8$  cells, usually about  $5 \times 10^6$  to  $5 \times 10^7$  cells.

The number and temporal spacings of the inoculations will be sufficient to elicit the desired immunoprotective response against subsequent challenge by FIV. Usually, there will be at least two inoculations spaced at least one week apart, more usually being from two to 10 inoculations spaced over a period from two to thirty weeks. Often, a final inoculation may be administered at some longer interval following an initial series of administrations. The selection of optimum administration patterns for a particular vaccine formulation is well within the skill in the art.

Diagnostic tests for detecting the presence of FIV in biological samples may also be performed using polynucleotide probes. Such polynucleotide probes may be prepared based on the sequence of the viral genome. The length of the probe is not critical, but will usually comprise at least about 12 bases, more usually comprising at least about 16 bases, which are substantially complementary to a portion of the viral genome. The probe itself may be DNA or RNA, and the probe need not have perfect complementarity with the FIV genome, with one or two mismatched pairs being acceptable for probes up to 20 bases in length and three to five mismatched pairs in probes from 20 to 35 bases. The probes may be prepared synthetically, with suitable synthetic techniques having been described above, and will include a detectable label. Usually, the synthetic sequences are expanded in commonly available cloning vectors and suitable hosts in order to obtain large quantities. The expanded vectors may themselves be labelled for use as probes, or shorter fragments containing complementary strands may be excised and labelled. Methods for the preparation and utilization of nucleotide probes for diagnostic testing are described in Falkow et al. U.S. Pat. No. 4,358,535, the disclosure of which is incorporated herein by reference.

A variety of labels have been employed, including those which have been described above for use in immunoassays, particularly radionuclides. Suitable labels may be bound to the probe by a variety of techniques. Commonly employed is nick translation with  $\alpha$ -<sup>32</sup>P-dNTP terminal phosphate hydrolysis with alkaline phosphatase followed by 5'-end labelling with radioac-

tive<sup>32</sup>P employing 7-P-NTP and T4 polynucleotide kinase or 3'-end labelling with an  $\alpha$ -<sup>32</sup>P-dNPT and terminal deoxynucleotidyl transferase. Alternatively, nucleotides can be synthesized where one or more of the atoms present are replaced with a radioactive isotope, e.g., hydrogen with tritium. In addition, various linking groups can be employed. The terminal hydroxol can be esterified with inorganic acids, e.g., <sup>32</sup>P phosphate or <sup>14</sup>C organic acids, or else esterified with bifunctional reagents to provide other reactive groups to which labels can be linked.

The following examples are offered by way of illustration, not by way of limitation.

The experimental work described below relating to the development and use of cell lines FeT-1M, FeT-2D, FL-4, and FL-6 was performed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

#### EXPERIMENTAL

##### Materials and Methods

###### Cell Types

Cells used as the source of FIV were the Crandell feline kidney cell line (FIV-CRFK) and feline mixed fresh PBLs (FIV-FeT1). Both cell types were infected with the Petaluma strain of FIV (A.T.C.C. No. VR 2186; deposited on Aug. 5, 1987, in connection with parent application Ser. No. 07/089,700). The FIV-CRFK line grows as a monolayer, morphologically similar to uninfected CRFK cells (Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258 and Fabricant et al. (1971) J. Am. Vet. Med. Assoc. 158:976-980). FIV-FeT1 cells, like uninfected FeT1 cells (mixed peripheral blood lymphocyte (PBL) cells from specific pathogen free (SPF) cats), grow in suspension and require interleukin-2 (IL-2). The IL-2-independent feline leukocyte cell lines, FL-4 and FL-6, were derived from the FIV-FeT1 cells and also are suspension cells which spontaneously produce FIV.

###### Cell Cultures

All suspension cell lines used in this study (FeT1, FL-4, FL-6) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES (N-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid), 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$ M 2-mercaptoethanol. IL-2-dependent cells were supplemented with 100 U/ml of recombinant human IL-2 (Cetus Corporation, Emeryville, Calif.). The suspension cells were passaged at a cell concentration of  $0.5-4 \times 10^6$  cells/ml and recultured in fresh culture media twice a week. FIV-CRFK cells were cultured in media consisting of equal volumes of L-15 and Eagle's minimum essential media, 10% heat-inactivated FCS, and 50  $\mu$ g/ml gentamicin. All monolayer cells were passaged twice a week at an initial cell concentration of  $2 \times 10^6$  cells/ml. The FIV-infected tissue culture fluids (TCF) were harvested twice a week, spun at 3000 rpm for 1 hr to remove residual cells, and stored at -20° C. or -70° C. or at 5° C. for those scheduled to be used within 1-5 days. One ml samples of cell-free infected TCF were routinely tested for Mg<sup>++</sup>-dependent reverse transcriptase (RT) activity as a means of monitoring for FIV production. Infected TCF were also checked routinely for Mn<sup>++</sup>-dependent RT activity to ensure that the cultures were producing only Mg<sup>++</sup>-dependent feline retrovirus (i.e., FIV). The RT assay used poly(rA)oligo(dT<sub>12-18</sub>) as an exogenous tem-

plate primer, four different deoxyribonucleotide triphosphates, 20 mM KCl with Mg<sup>++</sup> for detecting FIV or 60 mM NaCl with Mn<sup>++</sup> for detecting Mn<sup>++</sup>-dependent viruses (such as FeLV) and 5  $\mu$ Ci (<sup>3</sup>H)TTP alone per sample (Rey et al. (1984) Biophys. Res. Commun. 121:126-133). Five  $\mu$ Ci of (<sup>3</sup>H)TTP gave an average total count of 450,000 cpm using scintillation fluid mixture (1 part xylene to 2 part Amersham biodegradable counting scintillant) on a Bechman LS250 scintillation counter. As a result, our RT values will be below 450,000 cpm/ml.

#### Development of IL-2-Independent FIV Producer Lines

IL-2-independent FIV producing cell lines were developed from an IL-2-dependent FIV-infected feline PBL line (FIV-FeT1). The process of gradual IL-2 depletion from the FIV-FeT1 cell line took extensive sub-culturing over a period of approximately three months. The depletion process entailed a gradual reduction of the percentage of IL-2 containing media from the culture in the following weekly sequence: 75%, 50%, 25%, 5% and 0% IL-2-containing media. During this period over 80% of the starting cultures which were depleted of IL-2 did not survive the procedure. Surviving cultures were placed in individual 2-cm<sup>2</sup> multiwells at a viable cell concentration of  $2 \times 10^6$  cells/ml/well. During this stage only three of starting 20 cultures survived and these cultures were expanded sequentially into 25-cm<sup>2</sup>, 75-cm<sup>2</sup>, and 175-cm<sup>2</sup> flasks. One of the cultures (FL-5) did not survive. RT assays were performed on the surviving two cultures (FL-4 and FL-6) during the expansion period as means of monitoring FIV production.

#### In Vitro Infectivity Studies

The clarified infectious TCF from FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells was filtered individually with 0.45  $\mu$ m sterile filters to remove residual cells. These FIV inocula were aliquoted into 8-ml samples, stored at -70° C. and samples of these frozen inocula were retested for RT activity prior to in vitro infectivity studies. In all studies, the frozen inocula were thawed at room temperature immediately prior to use. FIV-susceptible feline cells ( $1 \times 10^6$  cells/ml) were infected with FIV at RT activity of 30,000 cpm/ml. All of the FIV-susceptible cells used in this study, with the exception of uninfected CRFK, were IL-2-dependent lymphoid cells which grew in suspension and required no trypsinization for passage. The TCF of the infected test cultures was harvested twice a week and the cells were recultured in fresh culture media containing IL-2. The harvested TCF was routinely tested for RT activity.

#### In Vivo Infectivity Studies

Two specific pathogen free (SPF) cats, 11 months of age, were inoculated intraperitoneally (IP) with 2 ml of infectious TCF from either FL-4 or FL-6 cells. Infectious TCF from FL-4 or FL-6 cultures, having RT activities of 150,000 cpm/ml, was aliquoted and stored at -70° C. The frozen virus inocula were thawed at room temperature and filtered with 0.45  $\mu$ m Millipore filter just prior to the inoculation. Both the single freeze-thawing and the filtering procedure ensured that the inocula were free of viable cells. The cats were bled routinely to obtain serum for serological assays and PBLs for virus isolation. Virus isolation consisted of

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co-cultivating 2-10 $\times$ 10<sup>5</sup> cells/ml with equal number of FIV-susceptible uninfected FeT1 cells and monitoring the TCF from these cultures for six weeks by RT assay. The PBL were considered positive for FIV isolation when RT activity of >10,000 cpm/ml were detected in TCF from at least two consecutive harvest days. The RT activity of the TCF from co-culturing PBL from SPF cats with FeT1 cells was <2,500 cpm/ml.

#### FIV Purification

FIV from infected TCF was concentrated and purified by ultracentrifugation, first on a 10/50% (w,v) discontinuous sucrose gradient and then on a 10/50% continuous sucrose gradient (Pedersen et al. (1987) Science 235:790-793 and Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S). The virus purified by this procedure was used for comparing the biochemical properties of FIV derived from different culture preparations and as the viral substrate for the immunoblot assay. Immunoblot analyses of gradient purified FIV from different infected cell lines (FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells) demonstrated the presence of the envelope gp100 band in blots from all viral sources. However, one major difference observed during these studies was that the intensity of the gp100 band was always weaker on the immunoblots made from purified FIV of FIV-CRFK origin than from those produced by other infected cell lines. Consequently, more viral antigen from FIV-CRFK cells was needed on the blots to get comparable intensity at the envelope band.

#### Immunoblot Analysis

A modification of the immunoblot technique described by Carlson et al. was used (Carlson et al. (1985) JAMA 253:3405-3408). Serum samples from immunized or FIV-infected cats were diluted to 1:50 in Buffer 3 (0.15M sodium chloride, 0.001M ethylene diaminetetraacetic acid, 0.05M Tris base, 0.05% Tween 20, and 0.1% bovine serum albumin) and incubated with the virus blot strips in individual wells for 18 hours at 37° C. These blot strips were then processed using a modification of a previously described procedure (Yamamoto et al. (1988) supra.). Briefly, the strips were incubated individually in wells with biotinylated anti-cat IgG (Vector Laboratories, Burlingame, Calif.) for 30 min and washed three times with wash solution. The strips were then incubated individually with horseradish peroxidase Avidin D (Vector Laboratories) for 30 min. After extensive washing, the strips were incubated with a fresh substrate solution (0.05% diaminobenzidine, 400 µg/ml NiCl<sub>2</sub> and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.1M Tris buffer, pH 7.4) at room temperature. After establishment of visible bands the reactions were stopped with excess distilled H<sub>2</sub>O, and the strips were then dried.

#### FIV p28 Assay

The FIV core protein p28 was detected by an enzyme-linked immunoabsorbent assay (ELISA) using two different monoclonal antibodies. A1 and B1 mAbs, to FIV p28 as either capture or substrate-reactive antibodies, respectively. Reactivity of both mAbs to FIV p28 antigen was confirmed by immunoblot analysis. The capture antibody (mAb A1) was coated on the plate overnight with bicarbonate buffer (pH 9.6) and washed once before its use. Serum samples to be tested were diluted in Buffer 3 and then incubated in the coated wells for 30 min at 37° C. The wells were

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washed six times with washing buffer, incubated with biotinylated mAb B1 for 30 min at 37° C., washed six times more, and then incubated with horseradish peroxidase Avidin D for 15 min. The wells were washed extensively again and finally incubated with substrate solution (0.005% tetramethylbenzidine and 0.015% H<sub>2</sub>O<sub>2</sub> in 0.96% citric acid solution) at room temperature. The reactions were stopped with 1M sulfuric acid solution upon establishment of a visible color reaction in the sequentially diluted standards consisting of purified FIV from pooled FIV-CRFK and FIV-FeT1 preparations.

#### Characterization of FL-4 and FL-6 Cell Lines

The phenotypic profiles of the feline cells were determined by fluorescence activated cell sorter (FACS) analysis using characterized monoclonal antibodies to feline CD4 (Fel 7), CD8 (FT2), pan T-cell, and to feline light chain and µ heavy chain specific (ACS) markers (Ackley et al. (1990) supra.; Ackley et al. (1990) supra.; Klotz et al. (1986) J. Immunol. 136:2510-2516; and Klotz et al. (1985) J. Immunol. 134:95-99). The cells were tested for mycoplasma using two different procedures both performed by Bionique Laboratories, Inc. The first procedure consisted of the direct DNA-fluorochrome staining of the cells for mycoplasma. The second procedure involved passaging test cells onto indicator cells which were then DNA/fluorochrome stained for mycoplasma. Detection of FeLV p27 core antigen was performed using the p27 antigen ELISA assay (Lutz et al. (1983) J. Immunol. Methods 56:209-220). Polymerase chain reaction (PCR) was used to test for the presence of FeLV provirus DNA. Briefly, a pair of primer sequences from the U3 region of the FeLV LTR were chosen so as to avoid the possibility of overlap with endogenous sequences of FeLV. The sequences of the two oligonucleotides primers used for PCR were 14 base pairs (bp 24 to 37) and 17 base pairs (bp 239 to 255) long. This enabled us to amplify a sequence of 232 base pairs for which we prepared a 25 base pair probe (bp 203 to 227) labeled with <sup>32</sup>P for identification by Southern blotting. The indirect fluorescent antibody assay to detect feline syncytium-forming virus (FeSFV) was performed as described previously (Pedersen et al. (1987) supra.; Yamamoto et al. (1988) supra.; and Yamamoto et al. (1989) J. Am. Vet. Med. Assoc. 194:213-220).

#### Immunogenicity of FIV Produced from FL-4 and FL-6 Cells

Eighteen SPF cats, 4-6 months of age, were used in these studies. Some of these cats were previously exposed to feline herpes virus (FHV)(A.T.C.C. C-27 strain) and the cats were free of FHV symptoms two-weeks prior to and during immunization. Three of the cats were immunized four times with 200 µg of inactivated FIV (inactivated whole virus) particles that were produced by pelleting cell-free TCF of FL-4 cells. An additional three cats were immunized eight times with 20 µm of inactivated whole virus. Seven cats were immunized either four or six times with 1 $\times$ 10<sup>7</sup> cells per dose of inactivated FL-6 or FL-4 cells (inactivated whole cell-virus), respectively. The pelleted virus and the infected cells were each inactivated with 1.25% paraformaldehyde, dialyzed against PBS, and then combined with adjuvant just prior to immunization. The adjuvants used were either threonyl muramyl dipeptide (MDP) (Byars et al. (1987) Vaccine 5:223-228)

or a combination of Freund's complete and incomplete adjuvants. Control cats were immunized with either uninfected FeT1 cells with adjuvant or diluent with adjuvant. All cats were immunized at two week intervals for a total of four or eight immunizations, unless stated otherwise.

## RESULTS

### Development of IL-2-Independent FIV-Producing Cell Lines

The development of IL-2-independent cell lines from FIV infected mixed PBLS (FIV-FeT1 cells) entailed the gradual depletion of IL-2 from the cultures. Only two out of 20 cultures, FL-4 and FL-6, survived the depletion process. Significant RT titers (100,000-400,000 cpm/ml), Mg<sup>++</sup> cation-dependent, were detected in these cultures during the expansion and large scale-production stage. Electron microscopy demonstrated numerous typical lentivirus particles in these cultures (data not shown).

The growth rates of these cell lines were compared to those of FIV-FeT1 and FIV-CRFK. The viable cell doubling time for FL-6 was found to be approximately 24 hours, whereas the doubling time for FL-6 was found to be approximately 24 hrs, whereas the doubling time for FL-4 was approximately 48 hrs. Both cell lines grew at an exponential rate. From a starting cell concentration of  $5 \times 10^5$  cells/ml, peak viable cell counts were observed after 3-4 days of culturing. Viability of the cells present in these cultures ranged from 70 to 90% over the four day culturing period. The number of syncytial cells in the FL-4 and FL-6 cultures was less than 0.1%. In comparison, the viability of FIV-FeT1 cells was only 55 to 65% after 1 day of culture, which may be attributed to dependence on IL-2. In our hands, a majority of the IL-2-dependent feline (FeT1, FeT2) and murine (HT-2C, CTLL-2) lymphoid cell lines have similar viability profiles. In order to evaluate the correlation between cell growth and virus production, samples from different harvest days were assayed for RT activity (FIG. 1). At a starting cell concentration of  $5 \times 10^5$  cells/ml, peak RT titers were observed on Day 4 of culture. Based on the number of cells present on Day 4, FL-4 cells produced the highest and FIV-CRFK the lowest RT activity.

### Characterization of the FL-4 and FL-6 Cell Lines

The phenotypic profiles of FL-4 and FL-6 cells were determined by flow cytometric analysis using monoclonal antibodies (mAb) to feline CD4 (fel 7), CD8 (FT2), pan T-cell (42) markers (Ackley et al. (1990) J. Virol. 64:5652-5655; Carlson et al. (1985) supra.; Ackley et al. (1990) supra.; and Klotz et al. (1986) supra.) and mAb that detect both feline immunoglobulin light chains and  $\mu$  heavy chain (ACS) (Klotz et al. (1985) supra.). The FACS profiles demonstrated that FL-4 cells were CD4 $\pm$ , CD8 $+$ , and Pan-T $+$  whereas FL-6 cells were CD4 $-$ , CD8 $\pm$ , and Pan-T $+$ . Both cell lines were negative for surface IgM and  $\lambda$  and  $\kappa$  light chains. It should be noted that both CD4 and CD8 antigens were lost in cultures maintained for several months. FL-4 and FL-6 cells were >95% positive by IFA for surface FIV antigen expression using polyclonal antibodies to FIV (Table 1). Additional tests were performed to ensure that these cells were free of known contaminants which could limit their use. The results are summarized in Table 1. The two cell lines were mycoplasma-free both by direct DNA/fluoro-

chrome stain and indirectly by passaging onto indicator cells prior to staining. Furthermore, FL-4 and FL-6 cells were shown to be negative for FeLV core protein p27 expression by ELISA and for exogenous FeLV DNA by PCR. The cells were determined to be negative by IFA for feline syncytial-forming virus (FeSFV).

TABLE 1

Absence of Known Contaminants in FL-4 and FL-6 Cell Lines			
Micro-organism Tested	Infection Status	Antigen Detected	Method of Testing
FIV	+	viral RT <sup>a</sup>	Mg <sup>++</sup> -dependent RT
	+	viral antigens <sup>ab</sup>	Immunoblot analysis with FIV-positive serum.
	+	mature virion	Electron microscopy.
	+	whole cell	IFA with FIV-positive serum (> 95% positive).
20 FeLV	-	viral RT <sup>a</sup>	Mn <sup>++</sup> -dependent RT.
	-	viral core	ELISA.
	-	p27	
	-	mature virion	Electron microscopy.
25 FeSFV	-	proviral LTR sequence in cellular DNA	PCR and Southern blot.
	-	mature virion	Electron microscopy.
Mycoplasma	-	whole cell	IFA with FeSFV-positive serum.
	-	whole cell	Direct DNA/fluorochrome staining for mycoplasma.
	-	whole cell	Indirectly by staining indicator cells which were passaged with FL-4 and FL-6 cells.

<sup>a</sup>These tests were performed on the tissue culture fluid harvested from the FL-4 and FL-6 cell cultures.

<sup>b</sup>Purified virus was disrupted with 0.1% SDS prior to its use in immunoblot production, as described in Methods.

### FIV Production in FL-4 and FL-6 Cell Lines

The amount of FIV produced from the FL-4, FL-6, FIV-FeT1 and FIV-CRFK cell lines was determined by comparing the total protein and RT levels of FIV in different fractions from sucrose gradient preparations (data not shown). High titers of both RT activity and total protein were observed in FIV preparations from FL-4, FL-6, and FIV-FeT1 cells. The FIV-CRFK produced low titers of FIV as demonstrated by the low levels of both protein concentration and RT activity in the fractions. The three peak fractions of the gradient purified virus from each cell line were pooled and measured for total protein concentration, RT titer, and viral core protein (p28) concentration. The results demonstrated a direct correlation between the p28, RT and the total protein levels present in the purified virus preparations. The viral antigen profiles of the FIV produced by the various cell lines were also compared by immunoblot analysis. Different concentrations of purified virus from different cell sources were used as substrate antigen for immunoblot strips. These immunoblots were then reacted with a set concentration of FIV-seropositive cat sera and the banding patterns evaluated. The immunoblot profiles from FL-4 and FL-6 cells were similar to those of FIV-FeT1 and FIV-CRFK (data not shown). The intensity of the bands, especially the viral envelope and transmembrane glycoproteins, produced on immunoblots from FL-4, FL-6 and FIV-FeT1 viral

substrates was stronger than that from FIV-CRFK. In addition, immunoblots of significantly higher quality were produced from the larger quantity of purified virus obtained from the TCF of FL-4 and FL-6. Thus, these results further indicate that a larger amount of virus antigens was produced by the FL-4 and FL-6 cell lines than by the FIV-FeT1 and FIV-CRFK.

#### Characterization of FIV Produced From FL-4 and FL-6 Cells

The FIV produced from FL-4 and FL-6 cells was tested for its ability to infect FIV-susceptible cell lines (FIG. 3). Cell-free TCF from different infected cell lines was inoculated into various feline cell cultures at a set RT concentration of 30,000 cpm/ml. FIV from FIV-CRFK cells did not readily infect certain feline lymphoid cells, in particular thymus-derived cultures, as compared to the FIV from FL-4 and FIV-FeT1 cells. The FIV from FL-6 cells was also highly infectious to FIV-susceptible cell lines (data not shown). Next, the FIV preparations produced from FL-4 and FL-6 cells were tested for their ability to infect SPF cats (FIG. 4). One SPF cat each was inoculated IP with 2 ml of cell-free TCF from either FL-4 or FL-6 cells. Both cats developed antibodies to FIV within four weeks post-infection. By sixteen weeks post-infection, the immunoblot profiles of these sera demonstrated the presence of antibodies to the majority of viral core antigens, but not to the viral envelope or transmembrane glycoproteins. Both cats were positive for virus isolation from PBLs. These studies demonstrated that the virus preparations from the FL-4 and FL-6 cell lines were highly infectious in both *in vitro* and *in vivo* systems.

#### Immunogenicity of FIV Produced From FL-4 and FL-6 Cells

Immunization of four cats with the inactivated FL-4 cell preparations ( $1 \times 10^7$  cells) led to the production of FIV antibodies specific for the viral core protein p28 soon after the second immunization (FIG. 5A). Antibodies to other viral antigens were demonstrated only after the third or fourth immunization (FIG. 5A). Thus the development of the antibodies in immunized cats closely mimics the FIV antibody development in experimentally infected cats (Yamamoto et al. (1988) *supra* and Hosie et al. (1990) *supra*). Control cats immunized with uninfected FeT1 cell preparations did not develop viral antibodies over the duration of the six immunizations.

Six other cats were immunized with inactivated FL-4-produced virus (200 µg) or inactivated FL-6 cell ( $1 \times 10^7$  cells) preparations together with a combination of complete and incomplete Freund's adjuvant instead of MDP (FIG. 5B). Both inocula led to the production of antibodies specific to the viral p28 shortly after the second immunization. Two out of the three cats immunized with the inactivated virus preparation developed antibody responses to viral envelope, whereas all three cats immunized with the inactivated FL-6 cell preparation developed antibodies to the envelope shortly after the second immunization. When other cats were immunized with 20 µg of pelleted inactivated virus in MDP) per dose, two out of three cats developed antibodies to the viral envelope, but only after the sixth immunization (FIG. 5B). Furthermore,  $1 \times 10^7$  viable FL-4 or FL-6 cells released into the TCF approximately 10 µg equivalence of purified virus (by sucrose gradient method) or approximately 30 µg equivalence of crude pelleted

virus when at their peak production level (data not shown). These findings suggest that it is more practical to use inactivated whole infected cells as an immunogen than inactivated whole virus for development of antibodies to the viral envelope. No difference in antibody development to viral p28 was observed between the infected cell or cell-free virus immunogens. Thus, our studies demonstrate that cats immunized with inactivated whole FIV-infected cells generate higher FIV envelope antibody titers more rapidly and more consistently than those immunized with inactivated cell-free whole virus.

#### Vaccination with Inactivated Cell Lines and Post Vaccination Challenge

The fixed cell-virus vaccine consisted of FIV-FeT1 and FIV-FL-4 inactivated with paraformaldehyde. In each culture (which was subsequently inactivated) essentially 100% of the cells were productively infected with FIV and  $5 \times 10^7$  cells were required to obtain 100 µg of total viral protein. Analysis of the FIV-infected cells in both T-cell lines by immunoblot using serum from an FIV immunized cat and by Coomassie stain, showed that the vaccine preparations contained the env, gag and pol virion proteins and their precursors as well as some regulatory proteins and cellular proteins (data not shown). The adjuvant used was threonylmuramyl dipeptide (MDP) (Syntex SAF-A).

The infected cells were inactivated with 1.25% paraformaldehyde for 24 hrs and washed three times with phosphate buffered saline (PBS). The vaccine consisted of  $1 \times 10^7$  inactivated FIV-FeT1 cells (Group 1A) or FIV-FL-4 cells (Group 1B) mixed with 250 µg of MDP. All cats in Group 1 were specific pathogen free (SPF) cats of 4-6 months of age, which were previously exposed to feline herpes virus (FHV C-27 strain) and were free of FHV symptoms two weeks prior to and during immunization. Ten control cats were immunized with either uninfected FeT1 cells with MDP (Group 1C) or MDP alone (Group 1D). All cats were challenged IP with 10 animal infectious doses (AID<sub>100</sub>) of homologous FIV petaluma strain two weeks after the final immunization. For the whole-virus vaccine, FIV was pelleted from the culture fluid of FIV-FL-4 cells, inactivated with 1.25% paraformaldehyde for 24 hours, and dialyzed extensively against PBS. Cats in Group 2A were SPF cats which were immunized with the inactivated FIV particles Group 2B cats received  $1 \times 10^7$  inactivated uninfected FeT1 cells mixed with the inactivated virus. Three additional SPF cats were immunized with either adjuvant (Cat #182) or diluent (Cats #55D and #55H). All cats were challenged with 10 ID FIV (homologous strain) two weeks after the final immunization. Virus was isolated from PBL and bone marrow cells by co-culturing with FIV-susceptible FeT1 cells. PCR analysis was performed using the method previously described (Pedersen et al. (1989) *J. Virol.* 64:598-606).

Nine cats in total were vaccinated subcutaneously (SC) with  $1 \times 10^7$  cells mixed with MDP (250 µg) five times at two week intervals and a final boost was given two months later. Five cats received the FIV-FeT1 cells (Group 1A) and four cats received the FIV-FL-4 cells (Group B). Ten control cats were inoculated with the uninfected allogeneic T-cells mixed with adjuvant, or adjuvant alone (Groups 1C and 1D, Table 2). Both fixed cell-virus vaccines induced significant levels of FIV antibodies (1:5,000 to 1:50,000) after the first-boost

as detected by whole-virus ELISA (FIG. 6A). By immunoblot, all nine vaccinated cats showed antibody to the viral core protein p24 after the first boot and antibody to other viral antigens, including the envelope gp100, after the third or fourth immunization (FIG. 7). Antibody response to the cellular components of the vaccine was weak as determined by immunoblot reactivity to the fixed uninfected cells. FIV-neutralizing antibody titers of 1:800 to 1:1200 were reached after the final immunization; these titers were equal to or slightly higher than the titers observed in unvaccinated cats experimentally infected with FIV (FIG. 8A). No infectious virus was detected in the vaccinated cats prior to challenge. Control cats remained free of antiviral antibodies and of infectious virus during the immunization schedule. The results are summarized in Table 2.

Virus was recovered persistently after 5 weeks pc from the PBLs of one vaccinated cat (#178) and after 21 weeks pc from another vaccinated cat (#138). The PBLs of both animals were PCR positive at 21 weeks pc at which time infectious virus was isolated from their bone marrow. These two persistently infected cats showed a sudden rise in antibodies by ELISA at the time virus was recovered, and the antibodies remained high thereafter (FIG. 6A). Also, by immunoblot, both core and envelope antibodies persisted longer in these cats than in the protected cats. These late breakthroughs of infectious virus in cats that had previously appeared protected indicate that only time can confirm the absence of latent virus in the protected vaccines. At 27 weeks pc, the seven vaccine protected cats showed FIV specific cell mediated response (CMR) as

TABLE 2

Isolation of FIV from Vaccinated Cats Before and After FIV-challenge							
			GROUP CODE				
GROUP 1A	GROUP 1B	GROUP 1C	GROUP ID	GROUP 2A	GROUP 2B	GROUP 2C	
135,137,142, 150,209	145,224,138, 178	136,147,164, 214,227	175,215,270, 271,278	55B,55F,094	55C,55I,177	182,55D,55H	VACCINE TYPE
FIV-FeT1 Cells	FIV-FL-4 Cells	FeT1 Cells (uninfected)	Placebo	Whole Virus	Whole Virus + FeT1 Cells (uninfected)	Placebo	
<u>FIV ISOLATION</u> (Positive Cat #) <u>From PBL</u>							
Pre-challenge	—	—	—	—	—	—	—
2 weeks post- challenge (pc)	—	—	—	ND	ND	ND	ND
3 weeks pc	—	—	147,214,227	175,270,278	—	—	—
5 weeks pc	209	178	147,214,227	175,270,278	ND	ND	ND
7 weeks pc	—	178	ALL	ALL	—	55I	ALL
17 weeks pc	—	178	ALL	ALL	—	—	ALL
21 weeks pc	—	138,178	ALL	ALL	ND	ND	ND
26 weeks pc	—	138,178	ALL	ALL	ND	ND	ND
<u>From Bone Marrow</u>							
21 weeks pc	—	138,178	ALL	ALL	—	—	ALL
<u>PCR OF PBL</u>							
21 weeks pc	—	138,178	ALL	ALL	—	—	ALL
<u>TOTAL # INFECTED</u>	1/5	2/4	5/5	5/5	0/3	1/3	3/3
<u>TOTAL # CHALLENGED</u>	(Transient)	(Persistent)	(Persistent)	(Persistent)	(Transient)		

— Indicates negative result.

Number indicates positive result from a specific cat with corresponding identification number.

ALL indicates that all cats in the specific group are positive.

ND indicates not done.

Two weeks after the final immunization, all of the cats were challenged intraperitoneally (IP) with 10 animal infectious doses (AID<sub>100</sub>) of the homologous FIV strain. Starting at seven weeks post challenge (pc) all ten control cats seroconverted, antibody titers gradually increased, and virus was persistently isolated from their peripheral blood lymphocytes (PBLs) (Table 2). By contrast, a steady fall in antibodies occurred and virus could not be isolated from the PBLs of six of the nine vaccinated cats for ≥ 21 weeks pc. These six cats were also free of detectable virus at 17 weeks pc as measured by bone marrow culture and polymerase chain reaction (PCR) analysis of PBLs and bone marrow cells. They therefore seemed to be solidly protected without evidence of latent proviral DNA. In one of the vaccinated cats (#209), virus was recovered from PBLs only one occasion, at 5 weeks pc, after which it was no longer detectable in either the PBLs (by virus isolation and PCR) or the bone marrow cells (by virus isolation). Antibody levels decreased steadily in this animal. Therefore, this animal may also be protected.

measured by positive lymphocyte proliferation and IL-2 induction assays (FIGS. 9A and 9B) as well as a positive response to non-specific mitogens (data not shown). By contrast, the two persistently infected vaccines and all infected control cats showed a lack of cellular response to FIV while the non-specific mitogen response remained intact. Since these cats were not tested for CMR before challenge we do not know if they were genetically poor responders and therefore vulnerable to infection or whether these defects in CMI were the result of infection. These findings suggest that the fixed cell-virus vaccine had induced T-cell immunity in the apparently protected cats. The duration of memory T-cell and the possible vaccine induction of cytotoxic T-lymphocytes remain to be determined.

#### 55 65 66 Vaccination with Inactivated Whole Virus and Post Vaccination Challenge

The cell-free whole virus vaccine was prepared from FIV-FL-4. Virus released from this cell line in high titer

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( $5 \times 10^8$  cells produced 1 mg viral protein per litter) was pelleted, filtered ( $0.45 \mu\text{m}$ ), inactivated with paraformaldehyde, and given with a combination of Freund's complete and incomplete adjuvants. Analysis of the cell-free pelleted FIV preparation from the whole-virus vaccine by immunoblot using serum from an FIV immunized cat showed that this vaccine contained all of the viral antigens, although a lesser amount of env glycoproteins than was present in the fixed infected cell vaccine, and also a trace amount of cellular antigens (data not shown). Six cats were immunized SC with 200  $\mu\text{g}$  viral proteins given every two weeks  $\times 4$ . Three control cats received either adjuvant or diluent alone. Significant levels of FIV antibodies (1:20,000 to 1:35,000) detected by whole-virus ELISA were induced after the first boost (FIG. 6B). By immunoblot, core p24 antibodies developed in all cats after the first boost and envelope gp100 antibodies developed in five of six cats after the third immunization (FIG. 7). Just prior to challenge, neutralizing antibody titers to FIV of 1:100 to 1:600 were present in all vaccinees (FIG. 8B). All cats were free of infectious virus prior to challenge.

Two weeks after the final immunization, cats were challenged IP with 10 ID of FIV. PBL cultures became virus and PCR positive by seven weeks pc from the three controls (Table 2), whereas five of six vaccinated cats remained uninfected up to 14 weeks. The PBLs of one vaccinated cat (#551) were transiently infected at 7 weeks pc but were negative by virus isolation and PCR at 17 weeks pc. After challenge, gradual decreases in antibody titers were observed in all immunized and protected cats including the single transiently infected cat. These findings show that effective protection against FIV challenge infection can also be achieved with an inactivated whole-virus vaccine.

Both the fixed cell-virus and whole-virus vaccines appeared to give roughly equal protection against challenge infection. However, the whole-virus vaccine was less immunogenic and required about 20-fold more cells to produce the amount of viral protein required to raise antibody titers to the levels approximating those observed with the fixed cell-virus vaccine. Nevertheless, the neutralization titers were still several fold less with the cell-free virus vaccine. This difference can probably be attributed to the difference in adjuvant used and/or to the greater amount and integrity of viral antigens presented on infected cells as compared to cell-free virus (Yamamoto et al. (in the press) Interviro., and Hosie and Jarrett (1990) AIDS 4:215-220). The cell-

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virus vaccine may also have elicited an allogenic effect from the inclusion of other cellular antigens. However, a mixture of uninfected allogeneic (FeT1) cells and inactivated whole virus (Table 2, Group 2B) did not enhance the ELISA and neutralizing antibodies to FIV as compared to whole virus alone. This indicates that the expression of viral antigens on the infected cell apparently provides the most effective immunogenicity.

The specific viral proteins and specific immune responses that account for the vaccine protection observed are as yet uncertain. Viral envelope appears an essential determinant because, in another trial, cats immunized with an FIV Iscom vaccine that was deficient in envelope antigen failed to make gp120 antibody and were not protected against challenge infection with 20 ID<sub>50</sub> of homologous virus. The vaccines of the present invention probably achieved a minimal threshold of protection because, using a similar fixed cell-virus vaccine we were previously unable to protect against a higher challenge dose ( $5 \times 10^3$  ID) of virus (data not shown). Although neutralizing antibody would seem a logical mechanism, other means of vaccine protection, such as antibody dependent complement lysis or cellular cytotoxicity (ADCC) against cell-free virus or infected cells, may also contribute.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A vaccine against feline immunodeficiency virus infection comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, wherein said immunogen elicits an immune response protective against infection by FIV when administered to a susceptible host in an amount effective to elicit such response.

2. A method for protecting a susceptible cat against feline immunodeficiency virus infection, said method comprising administering to said cat a vaccine comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, in an amount effective to elicit an immune response protective against infection by FIV.

3. A vaccine composition as in claim 1, further comprising an adjuvant.

\* \* \* \* \*

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ATTACHMENT D

[Return To:](#)[USPTO Home Page](#)[Finance Home Page](#)**Maintenance Fee Statement****5275813**

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 11, "STAT" below.

If a maintenance fee payment is defective, the reason is indicated by code in column 11, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,275,813	184	1950		07/739,014	01/04/94	07/31/91	08	NO	PAID

ITEM NBR	ATTY DKT NUMBER
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1 2307U-237-3/

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**BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV  
(1A55.20)**

Date	Description
28-Aug-91	Submitted FIV KV license application.
28-Aug-91	Submitted new FIV KV Production Outline.
28-Aug-91	Submitted research report entitled "Feline Immunodeficiency Virus Vaccine: Immunogenicity Study Proposal for USDA Licensure".
15-Nov-91	USDA approved the Production Outline with comments & pen-and-ink changes.
3-Feb-92	USDA responded to research report/protocol stating they are not yet prepared to state that the proposed study can meet the intended goal.
15-Jun-99	Requested USDA to transfer this product to their inactive files as we are discontinuing our efforts to license the product. The 2 strain product had, to this point, contained a strain (FIV alternate subtype A infected cell line) which we decided in 1999 to replace with the present strain (FIV subtype D infected cell line). The FIV subtype A infected cell line strain in the discontinued project remains in the present product due to be licensed (15A5.21). Although the 1A55.20 licensing project was cancelled in 1999, its FIV subtype A infected cell line fraction is actually the basis on which our 2001 license for 15A5.21 will be built.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
4-May-98	Submitted FIV-KV license application.
1-May-98	Submitted new FIV-KV Production Outline.
4-May-98	Submitted FIV-KV subtype A infected cell line master cell stock qualification report (MCS & MCS+25) and 2008s. Requested TA# to submit to CVB-L and permission to transfer to production.
4-May-98	Submitted FIV-KV alternate subtype A cell line master cell stock qualification report (MCS & MCS+25). Requested TA# to submit to CVB-L and permission to transfer to Production.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the subtype A infected cell line to Production at our own risk.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the alternate subtype A cell line MCS to Production at our own risk.
13-Aug-98	Submitted efficacy protocol.
5-Nov-98	The license application has been filed.
1-Dec-98	The new Outline was approved w/ pen-and-ink changes & comments.
7-Jun-99	Letter (faxed) requesting permission to move virus fluid from R & D building to production room 520, bldg 115 to be killed by autoclaving.
9-Jun-99	Permission granted to move the virus fluid from R&D to Production. However, questions were raised with other CVB-IC personnel
2-Jul-99	Submitted letter for master seed/master cell stock qualification report for FIV subtype D infected cell line. Requested permission to submit cell line to CVB-L and transfer to our production department. Requested TA #.
15-Jul-99	7/15/99 received verbal permission to transfer FIV subtype D infected cell line to Production at our own risk & TA#7610 to submit MCS samples to CVB-L. Confirmation letter received on 7/23/99.
20-Jul-99	Submitted FIV subtype D infected cell line along with the X+25 to CVB-L for confirmatory testing.
20-Jul-99	Submitted subtype A infected cell line to CVB-L for confirmatory testing.
4-Aug-99	Responded to Renee's June 9, 1999, questions (approval was granted) in regard to our moving a bioreactor to Room 510 Building 115 for the purpose of autoclaving.
4-Aug-99	No response required to the questions in regard to moving a bioreactor to Room 510.
27-Aug-99	Submitted subtype A infected cell line samples for testing.
4-Oct-99	A revised efficacy protocol which includes sufficient detail to address USDA comments needs to be submitted for consideration by the CRT prior to initiation of the study.
12-Oct-99	Submitted a revised efficacy protocol in response to USDA's 10/4/99 letter prior to initiation of the study.
29-Dec-99	Approved the revised efficacy protocol for the purpose intended.
24-Jan-00	Authorization to ship inactivated FIV vaccines to Japan was not granted based on the info.i.e. VS Code ?, serial numbers.
14-Jan-00	Submitted letter to Dr. Carr requesting permission to ship two experimental inactivated FIV vaccines to Japan.
14-Feb-00	Submitted complete revision of Production Outline in response to USDA comments.
13-Mar-00	Submitted response to 1/24/00 letter regarding permission to ship FIV to Japan. Need to clarify if there is a VS Code, serial number, etc.
21-Mar-00	Approved the request to ship FIV inactivated vaccines to Japan with comments.
17-Apr-00	Submitted new FIV post inactivation virus testing Special Outline 217.
19-Apr-00	SO 217 returned unprocessed.
3-Jan-01	Submitted letter requesting permission to ship experimental vaccines of FIV & FIV/FeLV to Japan.
9-Jan-01	Authorization received to ship experimental products of FIV & FIV/FeLV to Japan.
16-Jan-01	Submitted new FIV post inactivation testing SO responding to USDA 2015 comments dated 4/19/00.
15-May-01	Submitted preliminary field safety trial protocol.
17-May-01	Submitted Immuno report & included a disk with statistical analysis.
17-May-01	Approved subtype A MCS X+25 infected cell line for use in production.
17-May-01	Approved subtype A infected cell line for use in production.
17-May-01	Approved the FIV subtype D infected cell line MCS & X+25 for use in production.
19-Jun-01	SO was approved w/ pen-and-ink changes and comments.
19-Jun-01	Submitted in-vitro report for FIV.
19-Jun-01	Approved the preliminary field trial protocol w/comments. A form for owners with adverse events/daily observations column with clear instructions. Our final report should state number of properly completed forms returned to cooperating veterinarians.
19-Jun-01	Approved the complete revision of the Outline (in response to USDA comments 2/14/00) with comments and pen-and-ink changes.
21-Jun-01	Obtained field trial authorization from state vets.
25-Jun-01	Submitted revised outline of production.
26-Jun-01	Submitted new SO 237 for ELISA potency test for FIV.
5-Jul-01	No response required to comments re: owners completing forms during field trial.
5-Jul-01	Submitted letter responding to comment re: owners completing forms during field trial study.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
16-Jul-01	Submitted request for field safety test w/ 2008s for 2 of the 3 prelicensing serials. (Notify AR, VA, MD, CA of any adverse reactions--see state letters for their individual request.)
23-Jul-01	State vet approvals received.
24-Jul-01	Submitted letter advising of corrected address for Missouri site (Dr. Roger Sifferman) and copies of state approvals.
25-Jul-01	7/16/01 2008s for Serials 129050A & 129051A are filed as satisfactory. Verbal authorization on 7/20/01 to initiate field safety trial and ship 2000 doses of each serial. Amended protocol is approved.
25-Jul-01	USDA responded to the 7/5/01 ltr re: owners completing forms & their response was daily observation records from cat owners will not be required. Field safety protocol is adequate.
25-Jul-01	The change in address for the Missouri site was noted by USDA.
22-Aug-01	Immuno report filed as satisfactory.
30-Aug-01	Submitted ltr to Dr. Ludemann re: additional investigator for state of CA.
4-Sep-01	Verbal approval to ship to the additional investigator for the state of CA.
20-Sep-01	Submitted 2008 for 3rd PLS and requested TA#.
24-Sep-01	Submitted documentation of the storage temp of the Reference vaccine to support a 5 yr dating for frozen References. Approval of serial 1475-07-090299 (full dose) is contingent upon documentation (ltr of 8/22/01).
2-Oct-01	Received verbal TA#8279 to submit samples of the 3 PLS.
2-Oct-01	USDA approved new SO 237 ELISA potency.
3-Oct-01	USDA approved the revised Production Outline w/ comments & pen-and-ink changes.
5-Oct-01	Submitted samples of the 3 PLS to CVB-L under TA#8279 for confirmatory testing.
15-Oct-01	Reference 1475-07-090299 is approved for use as a Reference vaccine for serial release testing. The in vitro report satisfies concerns re: storage conditions of the immuno/ref serial. Need to submit FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 and approval of this report is contingent upon review of the subtype A infected cell line reaction w/ 1D9.
31-Oct-01	Submitted data for FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9.
2-Nov-01	The data submitted supporting the FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 was approved.
14-Nov-01	Submitted new label(s) (Spanish/English).
19-Nov-01	Submitted field trial report.
30-Nov-01	Changed FIV placebo vaccine lot# to 1516-62-031501. This lot was used in potency test validation and for all vaccine testing.
30-Nov-01	Submitted inactivation kinetics demonstrating that one inactivation procedure is sufficient for this virus per USDA 2015 comments dated 10/3/01.
4-Dec-01	Submitted "Supplemental Data - Addendum to the Demonstration of the Safety of FDAH's FIV Vaccine, KV in Cats Under Field Conditions". This should complete our submission for the field trial.
13-Dec-01	Submitted complete revision of SO 217.
18-Dec-01	Submitted results of study of experimental vaccines sent to Japan to CVB-L.
27-Dec-01	USDA approved the FIV placebo vaccine lot change subt'd on 11/30/01.
7-Jan-02	USDA approved the field trial report.
8-Jan-02	USDA approved the study of experimental vaccines sent to Japan.
9-Jan-02	USDA approved the inactivation kinetics.
9-Jan-02	CVB-L has completed satisfactory confirmatory testing of Prelicensing Serials 129050A, 129051A & 129052A.
4-Feb-02	Submitted new labels (FDAH requests the 10/22/01 & 11/14/01 submission be returned as sketches).
6-Feb-02	USDA approved labels submitted on 11/14/01 as sketches (per FDAH's request on 2/4/02).
21-Feb-02	Submitted new labels that replace sketches (FDAH requests the 2/4/02 submission be returned as sketches).
22-Feb-02	USDA approved labels submitted on 02/04/02 as sketches (per FDAH's request on 2/21/02).
25-Feb-02	Requested to ship up to 12 doses each of Serials 1749-20-122801 and 1749-25-122801 to Saitama, Japan.
13-Mar-02	USDA gave permission (verbal perm. given on 3/7/02) to ship up to 12 doses each of Serial Nos. 1749-20-122801 and 1749-25-122801 to Dr. Setsuo Arai of the Kitasato Institute, Saitama, Japan.
14-Mar-02	The Biological Product License was issued (we rec'd on 3/15/02).
14-Mar-02	USDA approved labels submitted on 2/21/02.
20-Mar-02	Submitted FDAH's press release to USDA.
21-Mar-02	Faxed a revised press release per telephone conversation between Dr. Ludemann & Dr. Steve Chu on 3/20/02.
22-Mar-02	USDA approved FDAH's press release subt'd on 3/20/02. This also confirms USDA's verbal approval on 3/21/02 (faxed) to add the patent acknowledgements for the University of California and the University of Florida.
22-Mar-02	Submitted efficacy report in 8-week-old kittens. (Cover ltr dated 4/1/02)
5-Apr-02	Submitted for USDA approval a press release to be distributed internationally.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
18-Apr-02	USDA approved the press release to be distributed internationally.
24-Apr-02	Submitted a quick synopsis on the FIV 6-month DOI. No response from USDA is required.
26-Apr-02	Submitted 2 separate letters ("Dear Distributor:" and "Dear Animal Health Industry Professional:") with advertising material attachments for USDA review and approval.
1-May-02	USDA gave verbal authorization (also confirmation letter dated 5/1/02) to distribute the advertising material identified as 2 letters.



ATTACHMENT B

United States  
Department of  
Agriculture

Marketing and  
Regulatory  
Programs

Animal and Plant  
Health Inspection  
Service

Veterinary Services

Center for Veterinary  
Biologics  
Suite 104  
510 South 17th Street  
Ames, IA 50010  
(515) 232-5785  
FAX (515) 232-7120

Federal Relay Service  
(Voice/TTY/ASCII/  
Spanish)  
1-800-877-8339



OVERNIGHT MAIL  
March 14, 2002

Ms. Madonna Carlson  
Fort Dodge Laboratories  
800 5th Street, NW  
P.O. Box 518  
Fort Dodge, IA 50501

RECEIVED

MAY 15 2002

OFFICE OF PETITIONS

Dear Ms. Carlson:

Enclosed is a new United States Veterinary Biological Product License issued this date to American Home Products Corporation, Establishment No. 112, authorizing production of the following:

Feline Immunodeficiency Virus Vaccine, Killed Virus, Code 15AS.21

Please note the restrictions under which this license is issued.

This U.S. Veterinary Biological Product License does not constitute a patent license. If this product or technology used in the manufacture of this product has been patented or is pending patent, the licensee should obtain a patent license from the patent owner.

If this license does not agree with your records, please return it to this office with your comments.

Sincerely,

Richard E. Hill, Jr., D.V.M.  
Director  
Center for Veterinary Biologics

Enclosure

cc: Bio Regulatory Affairs  
FILE: VS Code 15AS.21  
Product License Book

(see Bio Reg Notice #13 for  
distribution list)

RECEIVED

MAR 15 2002

BIOLOGICAL  
REGULATORY AFFAIRS



# United States Department of Agriculture

## UNITED STATES VETERINARY BIOLOGICAL PRODUCT LICENSE

Washington, D.C.

This is to certify that, pursuant to the terms of the Act of Congress approved March 4, 1913 (37 Stat. 832), governing the preparation, sale, barter, exchange, shipment, and importation of viruses, serums, toxins, and analogous products intended for use in the treatment of domestic animals, the person holding United States Veterinary Biologics Establishment License No. 112 is authorized to prepare in the facilities designated in the establishment license.

### FELINE IMMUNODEFICIENCY VIRUS VACCINE

Killed Virus

Code 15A5.21

Preparation shall be in accordance with the provisions of the Act, the regulations made thereunder, and additional restrictions or requirements when listed below.

1. For use by or under the supervision of a veterinarian.
2. Marketing and promotional materials must be submitted to the Center for Veterinary Biologics for review and approval prior to use.

This license is subject to termination as provided in the regulations made under the authority contained in said Act, and to suspension or revocation if the licensee violates or fails to comply with said Act or the regulations made thereunder.

March 14, 2002

Date

Michael A. Hough  
Director, Center for Veterinary Biologics  
Animal and Plant Health Inspection Service



US005275813A

**United States Patent [19]**

Yamamoto et al.

[11] Patent Number: 5,275,813

[45] Date of Patent: Jan. 4, 1994

## [54] METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

[75] Inventors: Janet K. Yamamoto, Hercules; Niels C. Pedersen, Winters, both of Calif.

[73] Assignee: The Regents of the University of California, Oakland, Calif.

[21] Appl. No.: 739,014

[22] Filed: Jul. 31, 1991

Yamamoto et al. (1988) Leukemia, Dec. Supp. 2:204S-215S.

Yamamoto et al. (1988) Am J. Vet. Res. 49:1246-1258.

Ackley et al. (1990) J. Virol. 64:5652-5655.

Olmstead et al. (1989) PNAS USA 86:2448-2452.

Olmstead et al. (1989) PNAS USA 86:8088-8092.

Talbott et al. (1989) PNAS USA 86:5743-5747.

Hosie and Jarrett (1990) AIDS 4:215-220.

Stott et al. (1990) Lancet 336:1538-1541.

Desrosiers et al. (1989) PNAS USA 86:6353-6357.

Murphrey-Corb et al. (1989) Science 246:1293-1297.

Carlson et al. (1990) AIDS Res. Human Retrovir. 6:1239-1246.

Berman et al. (1990) Nature 345:622-625.

## Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 618,030, Nov. 16, 1990, Pat. No. 5,037,753, which is a continuation of Ser. No. 89,700, Aug. 26, 1987, abandoned.

[51] Int. Cl. 5 .....

Primary Examiner—Christine M. Nucker

[52] U.S. Cl. .... 424/89

Assistant Examiner—D. Barnd

[58] Field of Search ..... 424/89; 435/240.2, 235.1

Attorney, Agent, or Firm—Townsend and Townsend Khourie and Crew

## [56] References Cited

## [57] ABSTRACT

## FOREIGN PATENT DOCUMENTS

WO90/13573 11/1990 PCT Int'l Appl. .... C07K 13/00

## OTHER PUBLICATIONS

Jarrett, O. et al. (1990) AIDS 4 (Suppl. 1):S163-165.  
 Berzofsky, J. A. et al. (1991), J. AIDS 4:451-459.  
 Pedersen et al. (1987) Science 235:790-793.  
 American Assoc. for Can. Res., May 23, 1987, Ab. No. 3337.  
 The 3rd Int'l. Conf. on AIDS, Jun. 1-5, 1987.  
 Yamamoto et al. Fed. Amer. Soc. for Experimental Biology, Apr. 2, 1987.

Compositions derived from a novel viral isolate designated feline immunodeficiency virus (FIV) include the whole virus, proteins, polypeptides and, polynucleotide sequences derived from the virus, and antibodies to antigenic sites on the virus. These compositions are useful in a variety of techniques for the detection of and vaccination against FIV. Detection methods disclosed include immunoassays for both the virus and antibodies to the virus, and the use of polynucleotide probes to detect the viral genome. Vaccines include both wholly and partially inactivated viruses inactivated cell lines expressing FIV antigens, and subunit vaccines. Whole, live virus is also useful as a model system for predicting the behavior of human immunodeficiency virus (HIV).

## 3 Claims, 11 Drawing Sheets

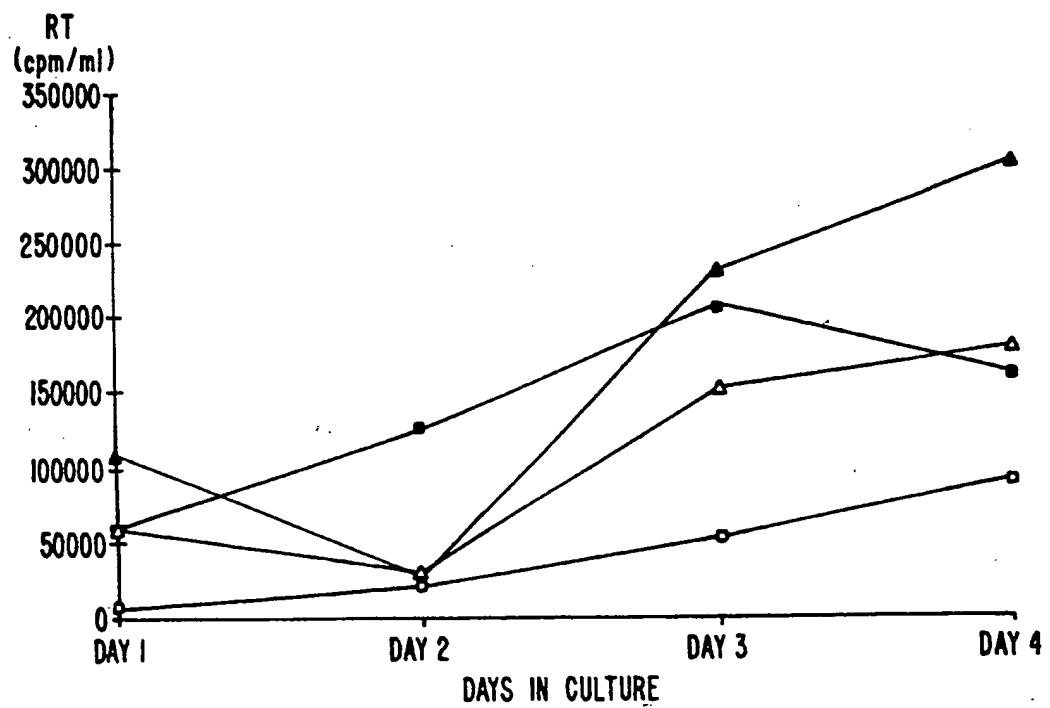
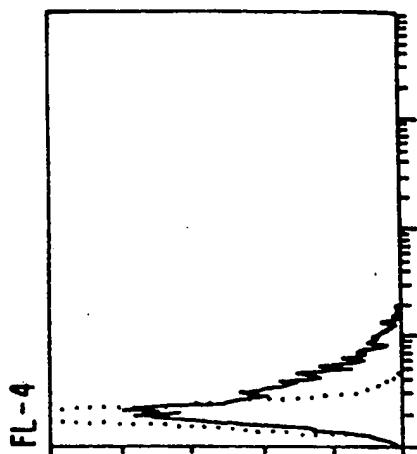
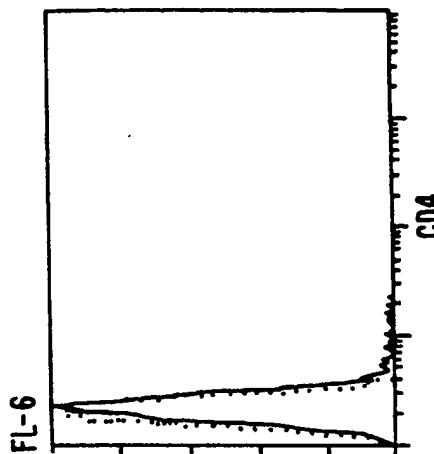
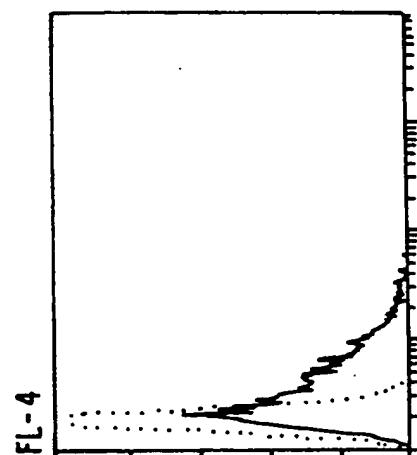
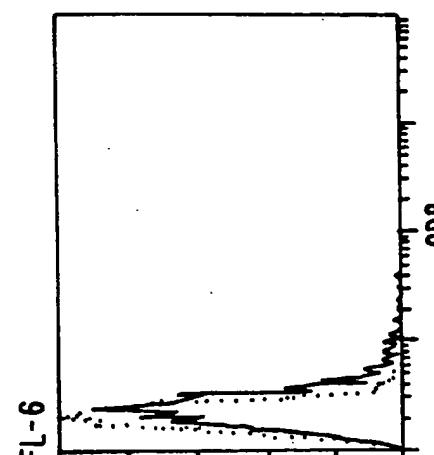
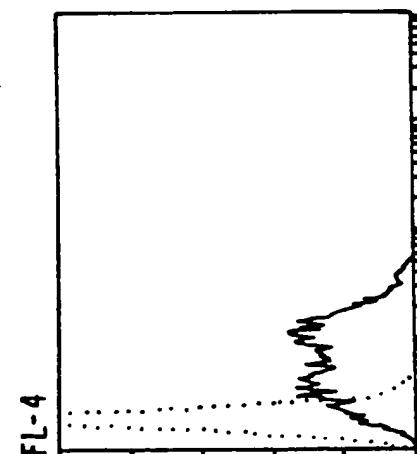
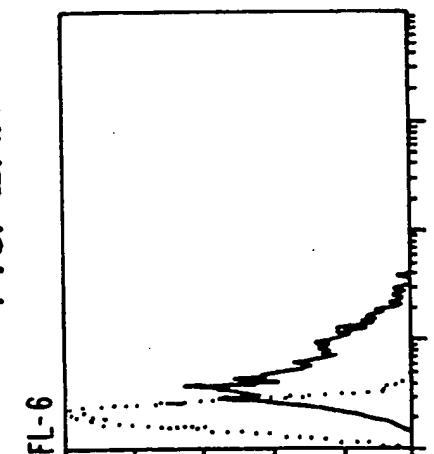
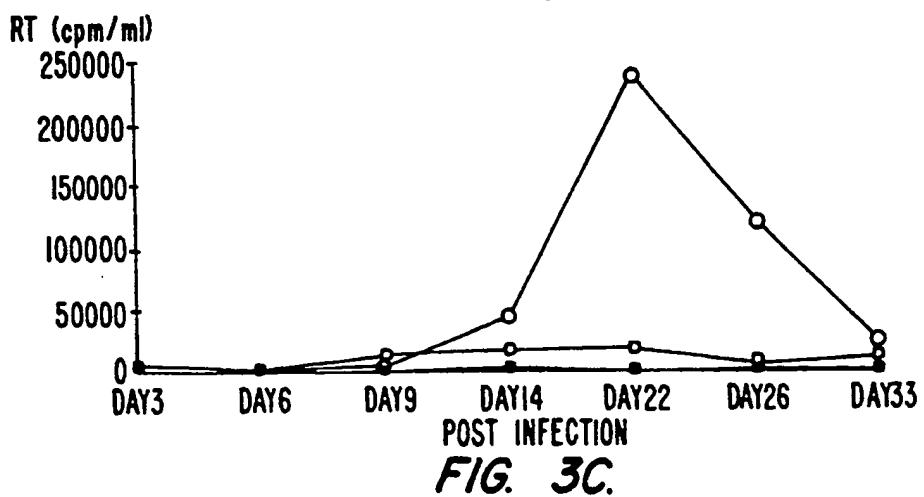
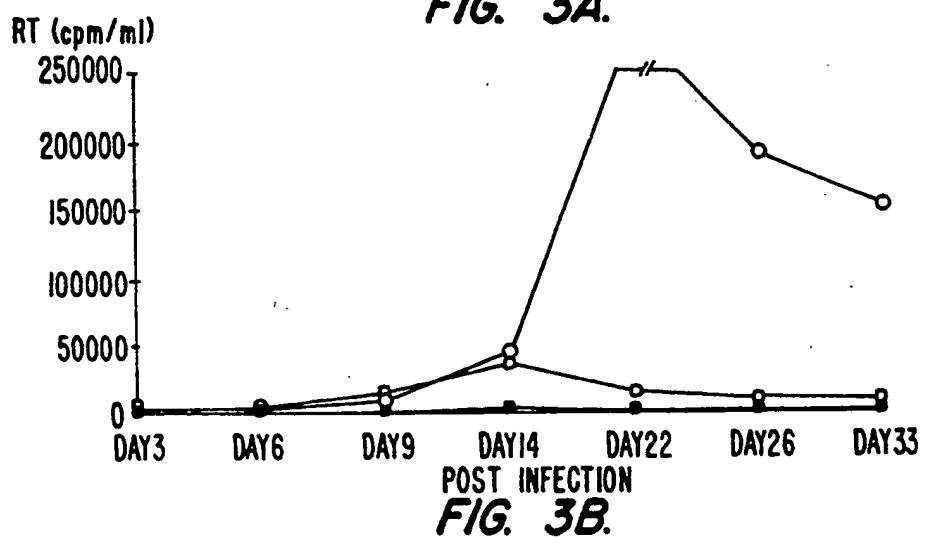
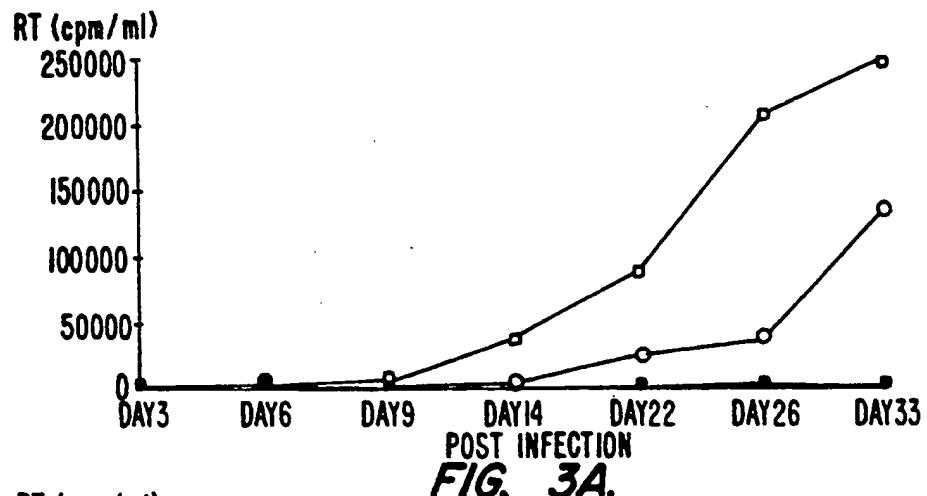
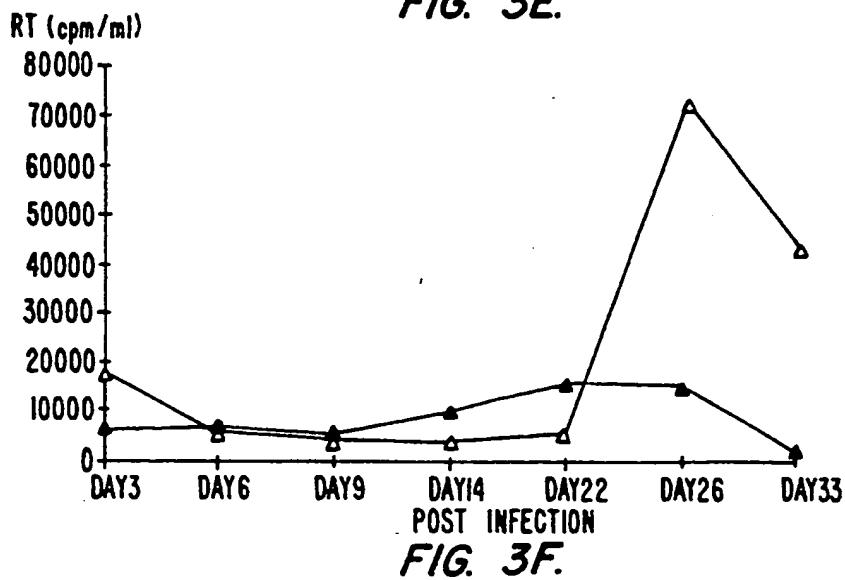
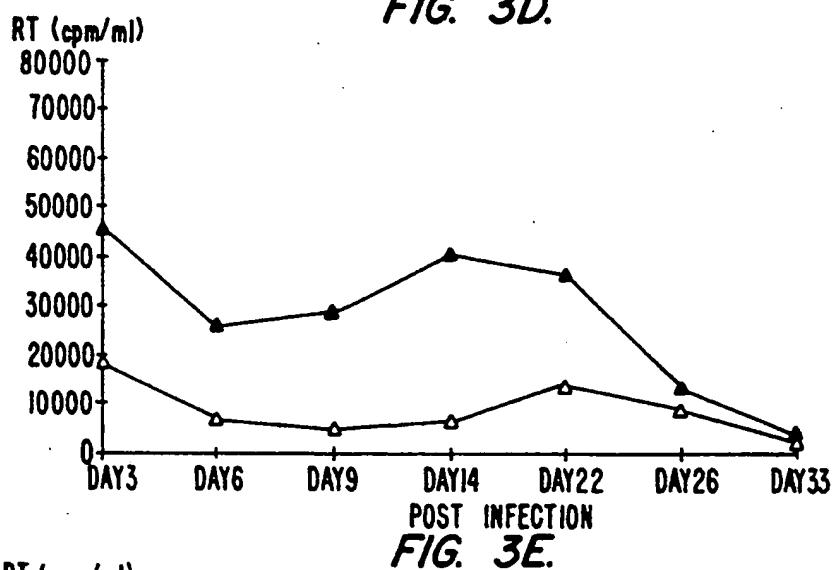
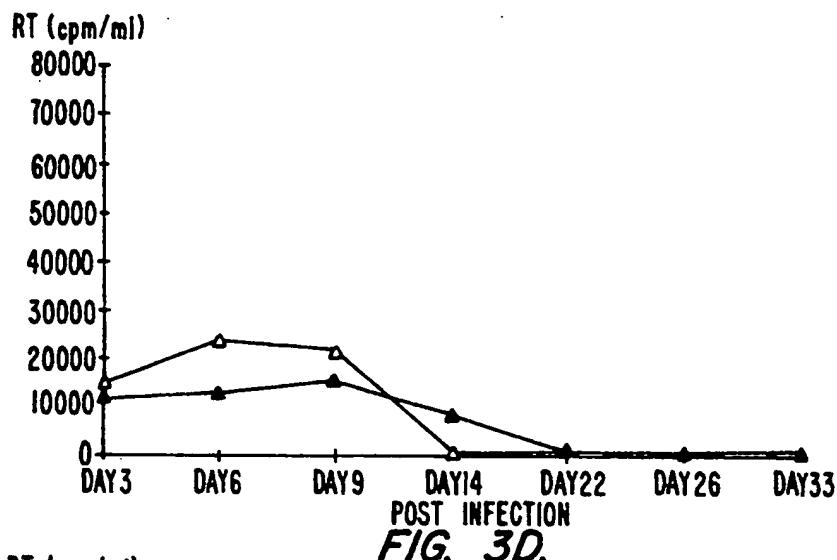


FIG. 1.

CD4  
FIG. 2C.CD4  
FIG. 2F.CD8  
FIG. 2B.CD8  
FIG. 2E.f42  
FIG. 2A.f42  
FIG. 2D.





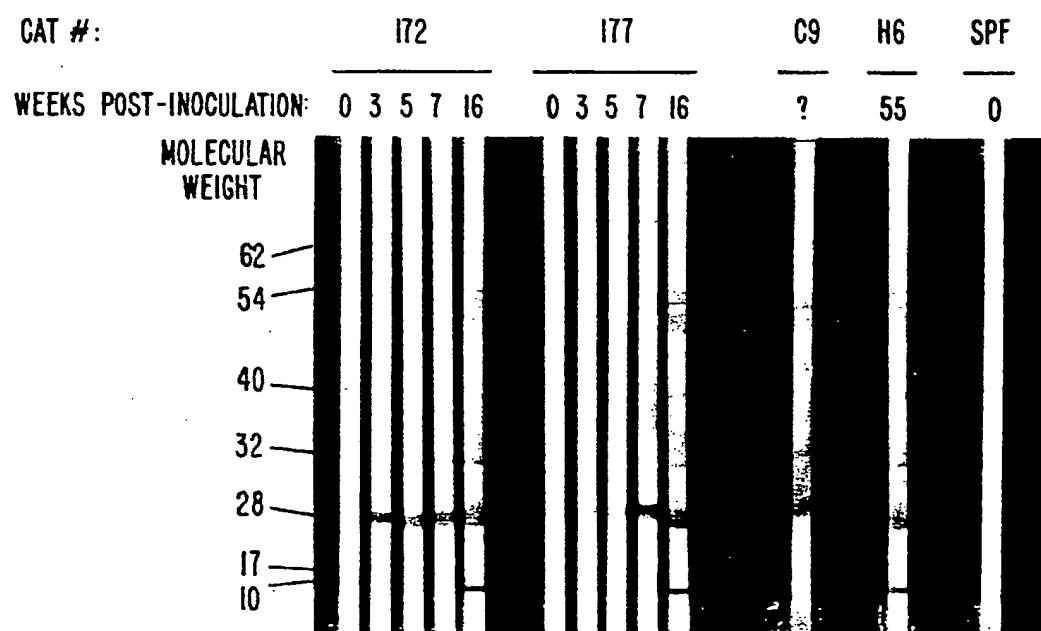
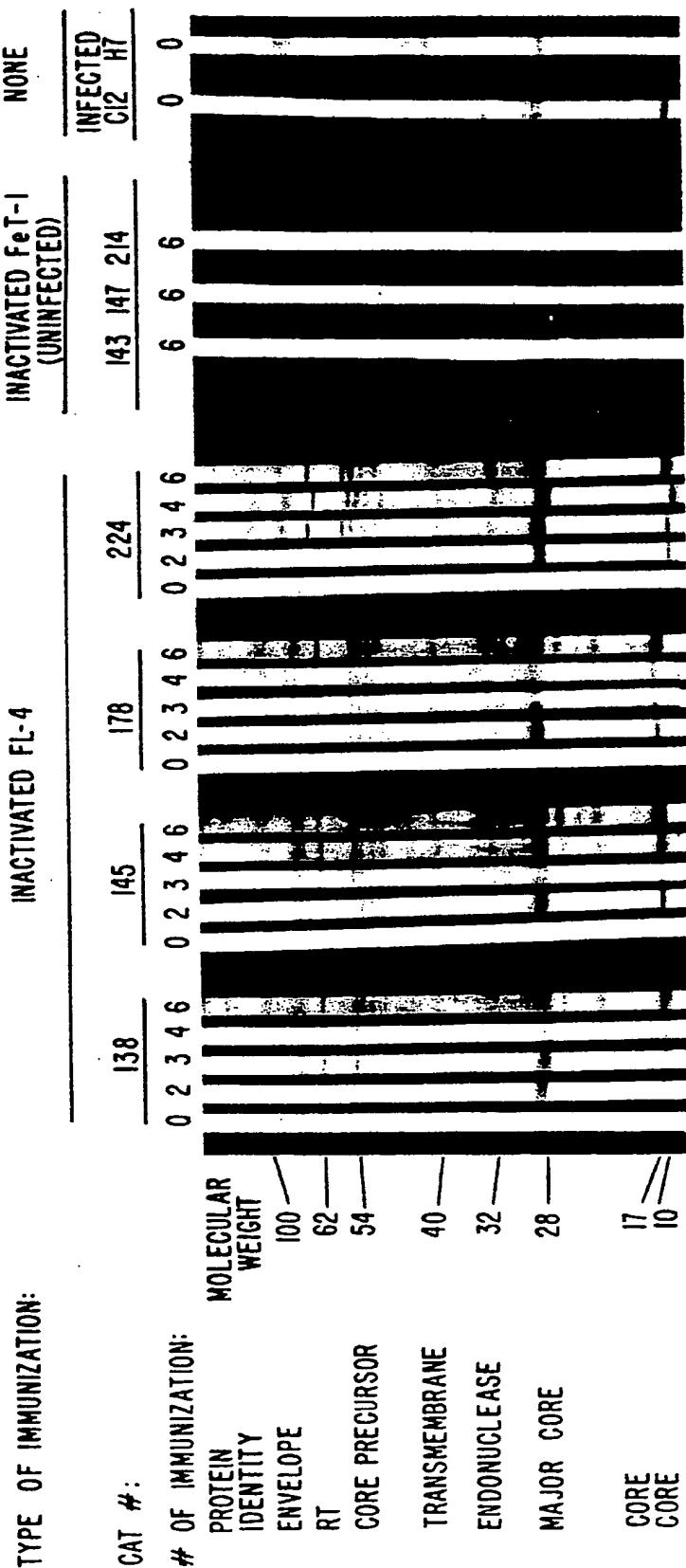


FIG. 4.



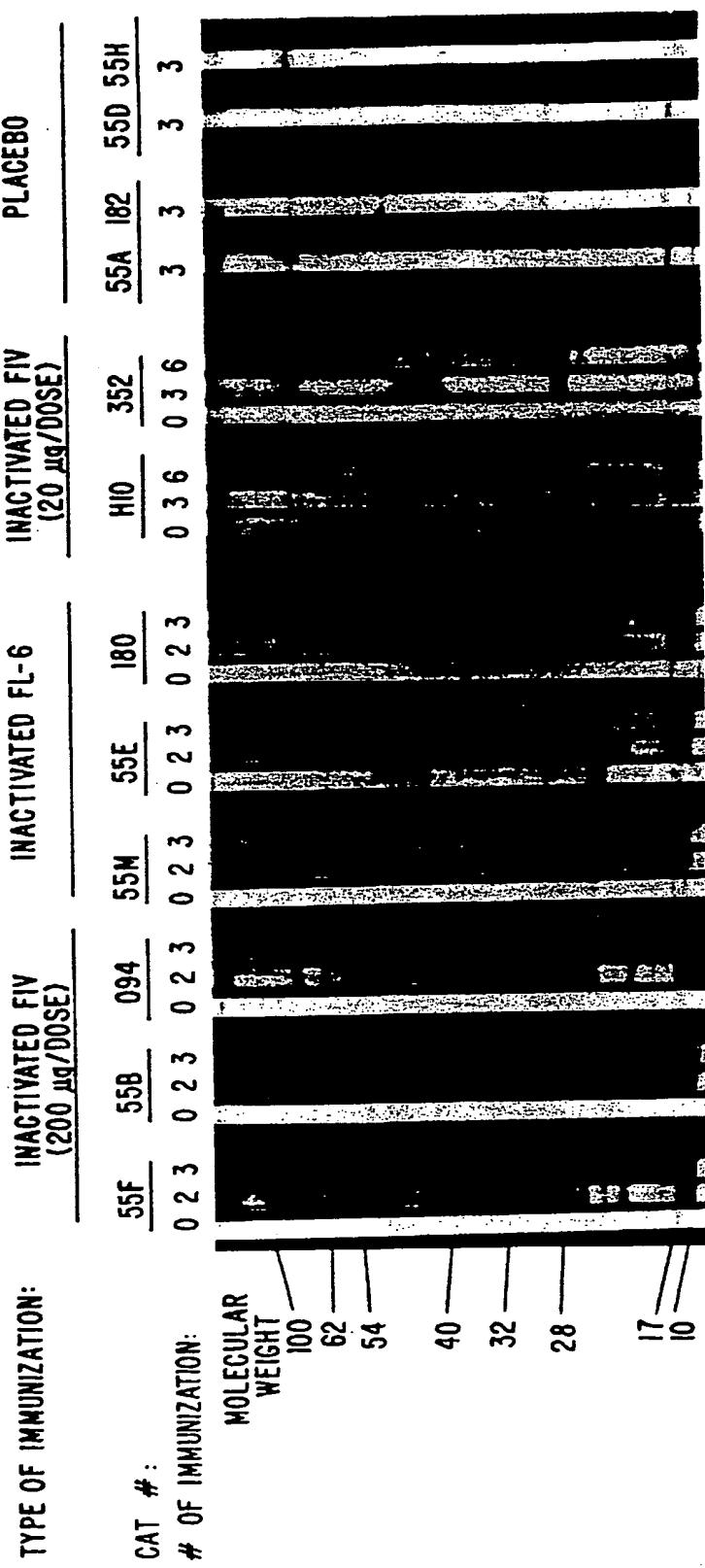
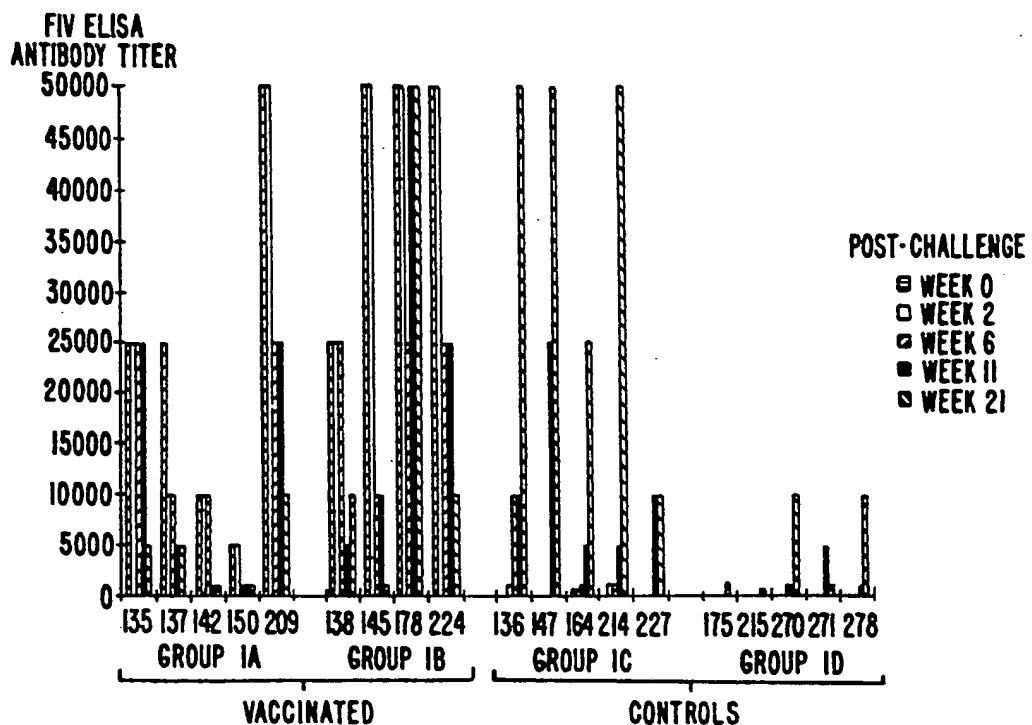
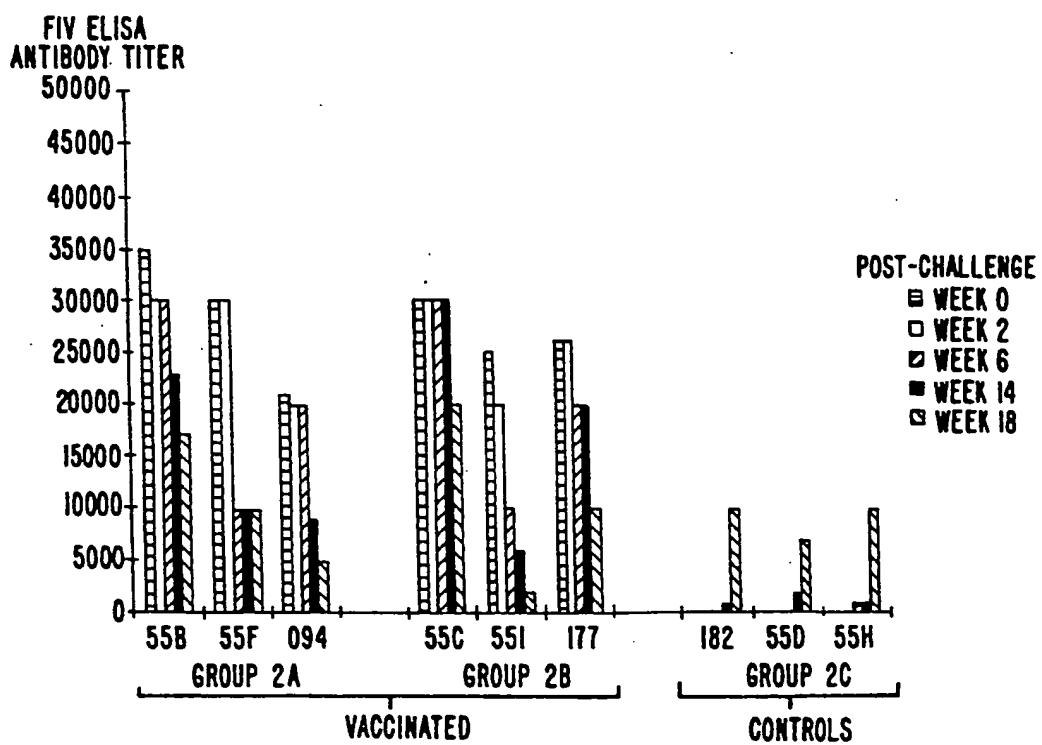


FIG. 5B.



*FIG. 6A.*



*FIG. 6B.*

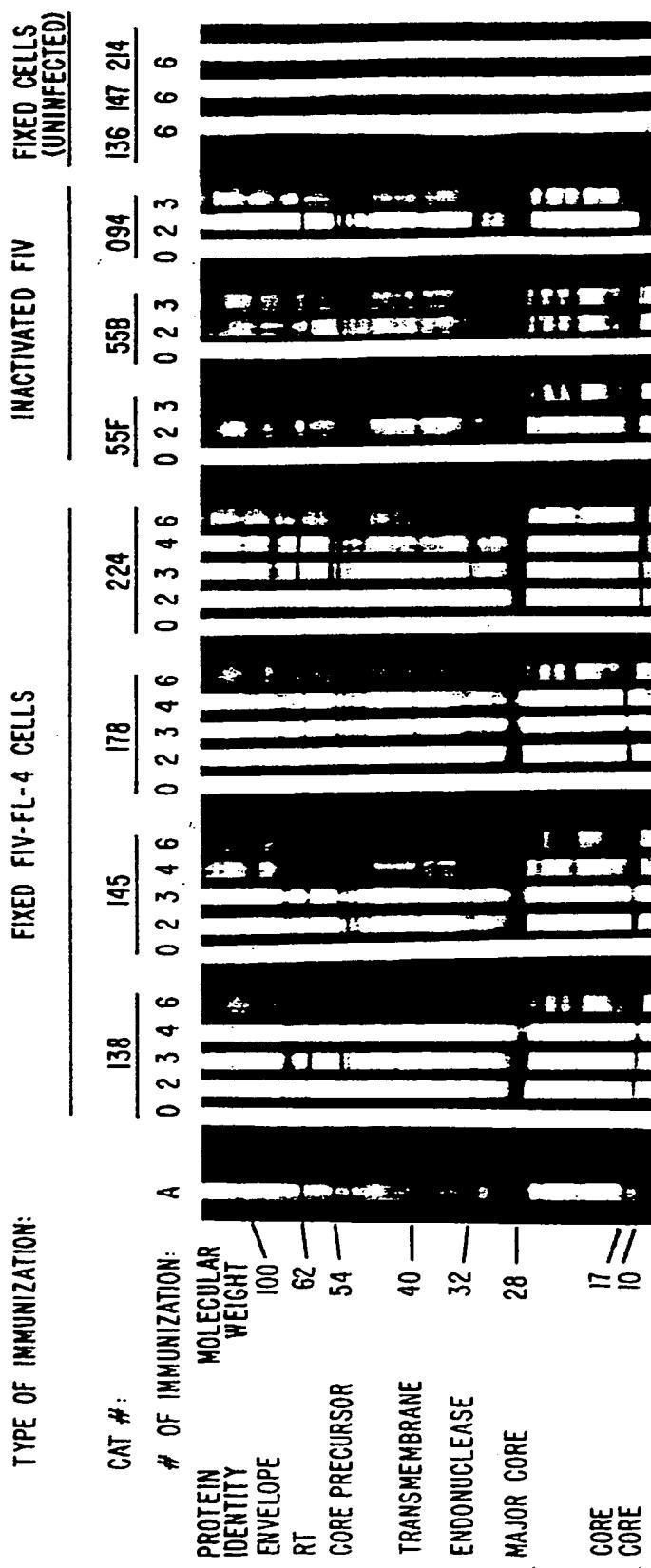


FIG. 7.

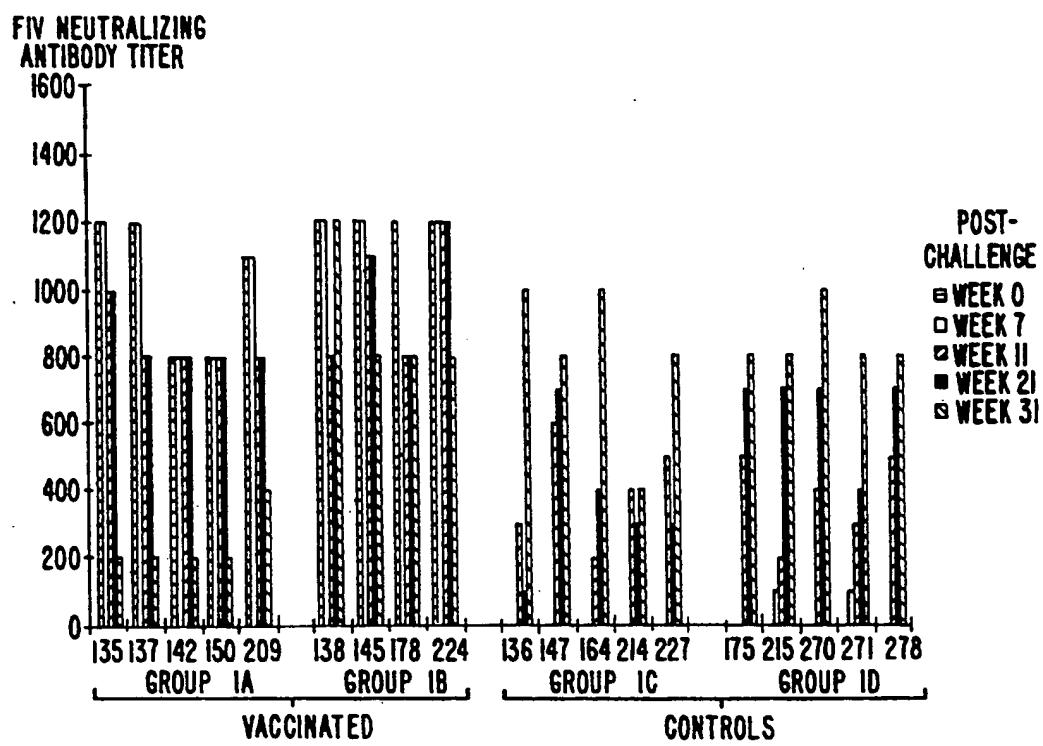


FIG. 8A.

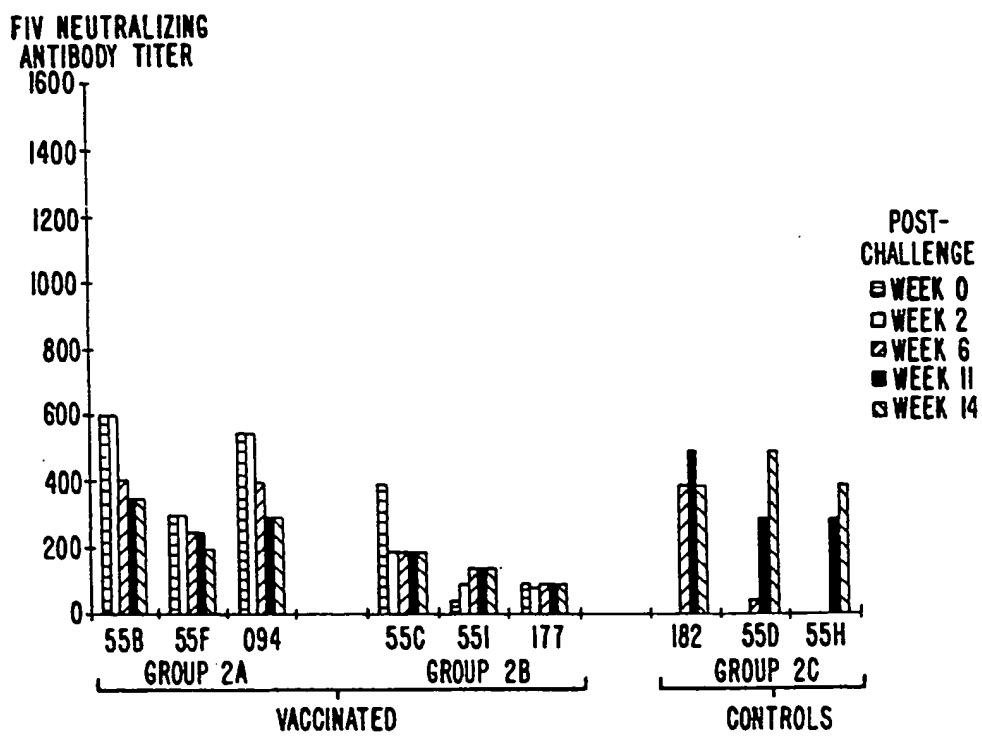


FIG. 8B.

## PROLIFERATION RESPONSE

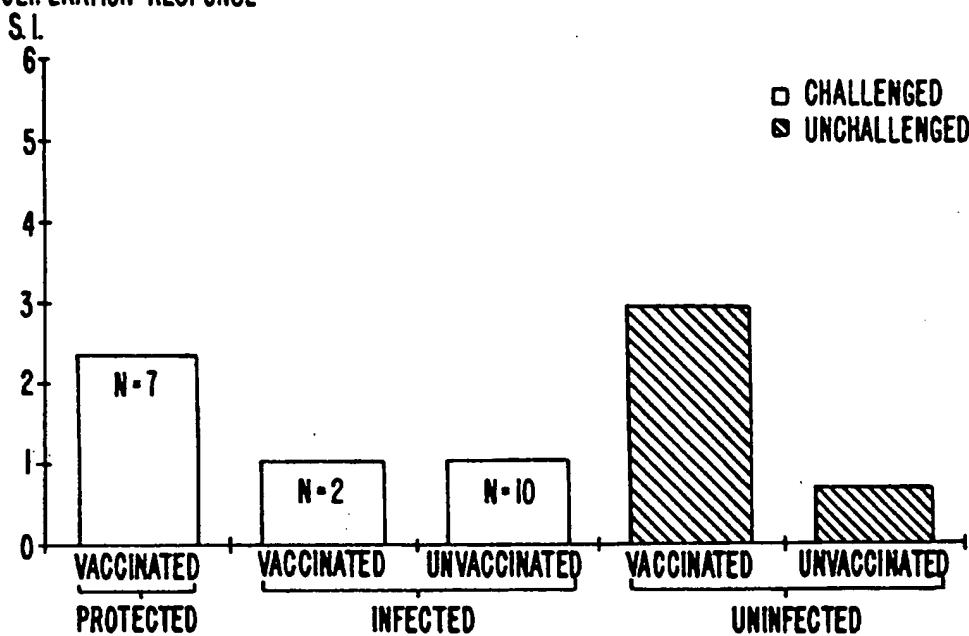


FIG. 9A.

## IL 2 INDUCTION

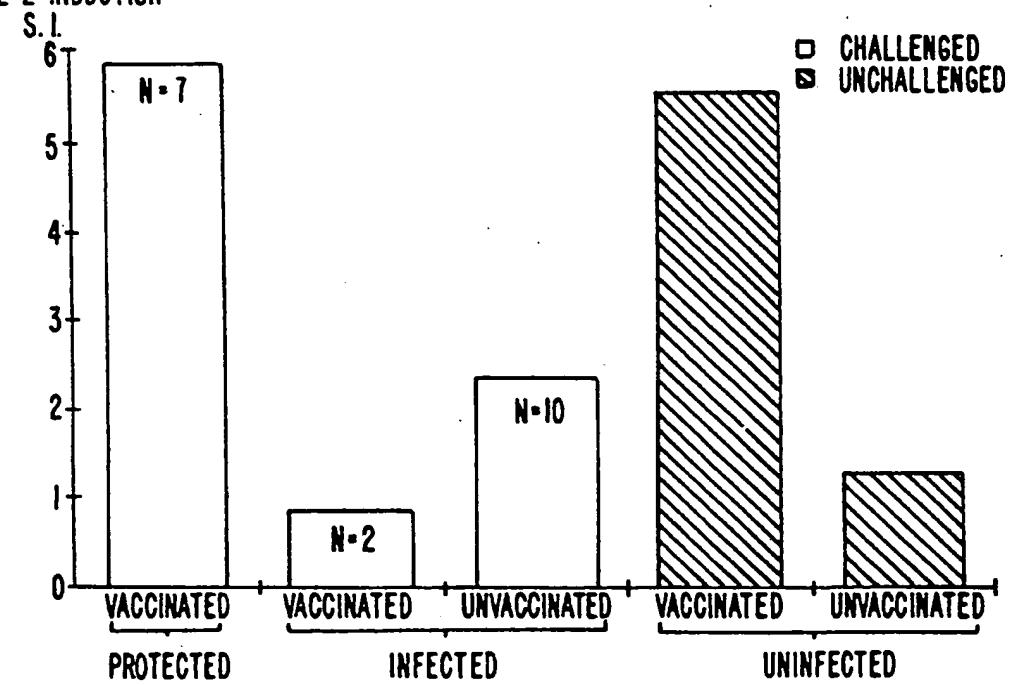


FIG. 9B.

## METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

This invention was made with Government support under Grant No. CA 39016 awarded by the National Institute of Health. The Government has certain rights in this invention.

The present invention is a continuation-in-part of application Ser. No. 07/618,030, filed on Nov. 16, 1990, now U.S. Pat. No. 5,037,753, which was a continuation of application Ser. No. 07/089,700, filed on Aug. 6, 1987, now abandoned. The disclosures of both these applications are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates generally to the detection and treatment of viral infection. More particularly, the invention relates to compositions and methods useful for the diagnosis of and vaccination against infection with a newly-discovered lymphotropic retrovirus, initially designated as feline T-lymphotropic lentivirus and presently designated feline immunodeficiency virus (FIV).

Domestic cats may become infected with several retroviruses, including feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncovirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immune-mediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

While immunodeficiency syndrome in cats has normally been associated with FeLV, immunodeficiency-like symptoms have been observed in cats which are seronegative for FeLV, usually without alternative explanation. It would be desirable to identify etiological agents other than FeLV which are responsible for causing immunodeficiency in cats. It would be particularly desirable to provide methods and compositions for the detection of and vaccination against such newly-identified etiological agents, and in particular, against FIV.

#### 2. Description of the Background Art

The discovery of feline T-lymphotropic lentivirus (now designated feline immunodeficiency virus) was first reported in Pedersen et al. (1987) Science 235:790-793 Abstracts concerning the discovery of the virus have been presented at the American Association for Cancer Research on May 23, 1987 (Abstract No. 3337); and The Third International Conference on Acquired Immune Deficiency Syndrome, Jun. 1-5, 1987. A poster concerning discovery of the virus was presented at a meeting of the Federation of American Society for Experimental Biology on April 2, 1987.

Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:8088-8092 and 86:4355-4360; and

Talbott et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:5743-5747. Hosie and Jarret (1990) AIDS 4:215-220, describes the serological response of cats infected with FIV.

A portion of the experimental data presented in this application was published in AIDS 1990 4 (Suppl. 1):S163-S165.

Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 336:1538-1541; Desrosiers et al. PNAS U.S.A. (1989) 86:6353-6357; Murphrey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

### SUMMARY OF THE INVENTION

Compositions and methods are provided for vaccination against a novel feline retrovirus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV). The compositions include vaccines comprising an immunogen capable of eliciting an immune response protective against infection by FIV when administered in an effective amount to a susceptible host. The immunogen will display determinant sites characteristic of the virus, such as those found on the major envelope and core proteins. The preferred immunogens include inactivated whole virus, attenuated whole virus, and inactivated cell lines infected with FIV and which express FIV antigens on their surface. Other immunogens which may find use include polypeptides which mimic the determinant sites, such as FIV peptides (to produce subunit vaccines), antiidiotype antibodies, and the like.

According to the method of the present invention, the vaccine compositions are administered to susceptible hosts, usually cats, in amounts effective to afford immunity against subsequent challenge by FIV. The vaccines may be administered by any conventional route, including subcutaneously, intramuscularly, and oranasally, and will usually be administered at least twice over intervals spaced-apart by one or more weeks to achieve the desired immunity.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: FL-4 ( $\Delta$ ), FL-6 ( $\Delta$ ), FIV-FeT1 ( $\blacksquare$ ), and FIV-CRFK ( $\square$ ) cells were seeded at  $5 \times 10^3$  cells/ml tested daily for the RT activity in their culture fluids. A gradual increase in RT activity was observed over the four days of culture, with peak RT titers detected on Day 4 for all cell cultures except FIV-FeT1 which had it on Day 3. Peak viable cell counts ( $1.0-2.25 \times 10^6$  cells/ml) were observed on Day 3 for all cell cultures except for the FIV-FeT1 culture which had its peak viable cell count ( $1.3 \times 10^6$  cells/ml) on Day 2. The percent cell viabilities during the four days of culturing were 75-90% for FIV-FL-4, 70-90% for FIV-FL-6, 70-80% for FIV-CRFK, and 55-65% for FIV-FeT1.

FIGS. 2A-2F: The FACS profiles of the surface phenotype of FL-4 (FIGS. 2A, 2B, and 2C) and FL-6 (FIGS. 2D, 2E, and 2F) were determined using characterized monoclonal antibodies to feline CD4 (Fel 7), CD8 (FT2), pan T-cell (F42) and the feline light chain and  $\mu$  heavy chain specific (AC5) markers. Both cell lines had cell populations which were positive for CD4 (FIGS. 2C and 2F), CD8 (FIGS. 2B and 2E), and pan T-cell (FIGS. 2A and 2D). Both FL-4 and FL-6 cells

tested negative by FACS analysis for surface B cell markers using monoclonal antibodies (AC5) (data not shown). The solid lines represent the FACS profiles of FL-4 and FL-6 cells and the dotted lines represent the FACS profiles of negative control cells. The percentages of FL-4 cells that were positive for CD4, CD8 and pan T-cell markers were 10%, 20%, and 80% respectively. The percentages of FL-6 cells that were positive for CD4, CD8 and pan T-cell markers were <8%, 11%, and 76%, respectively. Depending on the culture conditions, expression of CD4 and CD8 on the cell membrane can be decreased or eliminated. The abscissa represents fluorescence intensity and the ordinate represents relative cell number.

FIGS. 3A-3F: FIV from FL-4 (A,D), FIV-FeT1 (B,E) and FIV-CRFK (C,F) cells were tested for their infectivity on different feline PBLs (A,B,C) and feline thymocytes (D,E,F). Uninfected feline lymphoid cells used in this study were FeT1.1 (■), FeT1.2 (□), FeT1.3 (○), Thy1 (Δ), and Thy2 (▲). All of the FeT1 cells were derived from uninfected PBLs and Thy cells were primary thymocytes obtained from FIV-free kittens. FeT1.1, FeT1.2, and FeT1.3 were subclones of the uninfected FeT1 line, which was the precursor line for FIV-FeT1 cells. The percentage of cells that was positive for CD4 and CD8 markers was <2% and 5% for FeT1.1, <2% and <2% for FeT1.2, and <2% and 4% for FeT1.3, 54% and 4% for Thy 1, and 38% and <2% for Thy2, respectively. Interestingly, FIV from all cell lines were able to either transiently and persistently infect all lymphoid cells except for those from FeT1.2 cultures, whose cells also totally lacked the expression of both CD4 and CD8 markers. Another observation was that FIV from FL-4 and FIV-FeT1 cells infected thymocytes more rapidly but produced a lower titer of virus than those produced by FeT1.1 or FeT1.3 cells. The major difference between the thymocytes and the FeT1.1 or FeT1.3 cells was the large number of CD4+ cells present in the thymocyte cultures. Thus, this observation suggests that the rapid FIV infection of the thymocytes was correlated to the increased number of CD4+ cells.

FIG. 4: Immunoblot analysis was performed on the sera from cats inoculated with 2 ml of cell-free TCF (150,000 cpm/ml RT activity) from either FL-4 (Cat #172) or FL-6 (Cat #177) cultures. The FIV antibody development in these cats was similar to the progression observed previously in SPF cats inoculated with plasma or blood from FIV-infected cats or with TCF from primary PBL cultures (Yamamoto et al., (1988) *supra*). Their immunoblot profiles at 16 weeks post-inoculation (pi) resembled those of sera from naturally (Cat #C9) or experimentally (Cat #H6) infected cats. The PBLs from these cats at 10 weeks pi were positive for FIV by virus isolation (data not shown). Both immunoblot and virus isolation results demonstrate that these cats were infected with FIV.

FIGS. 5A and 5B: The immunogenicity of the FIV produced from our FIV-infected cell lines was evaluated in cats. The reactivities of the antibodies produced in cats immunized with either inactivated FL-4 (A) or FL-6 (B) cells or with inactivated FL-4-produced virus (B) were determined by immunoblot analysis. Cats were immunized six-times with inactivated FL-4 or uninfected FeT1 cells and their serum immunoblot profiles were compared to those of serum from cats naturally (Cat #C12) or experimentally (Cat #H7) infected with FIV (A). Cats were also immunized four-times with

inactivated FIV (produced by FL-4 cells) and with inactivated FL-6 cells (B) and evaluated similarly. In the last study, cats were immunized eight-times with 20 µg per dose of inactivated FIV and these results presented. All immunizations were done at two week intervals except for the final interval between the fifth and sixth immunizations in the first study. The adjuvants used were either MDP (A) or a combination of Freund's complete and incomplete adjuvants (B). The predicted molecular masses of the FIV proteins, derived from nucleotide sequence analysis (Olmstead et al. (1989) *supra*, and Talbott et al. (1989) *PNAS U.S.A.* 86:5743-5747), have been identified as: 24.5-25.1 kD for major core protein, 14.7-14.9 kD for N-terminal gag protein (minor core protein), 9.5-9.6 kD for nucleocapsid protein (minor core protein), 49.2-49.5 kD for gag precursor protein (core precursor), 61.5 kD for reverse transcriptase (RT), 30.7 kD for endonuclease, 100 kD for major envelope glycoprotein (outer membrane), 36 kD for transmembrane glycoprotein, and 140 kD for precursor envelope glycoprotein. The banding profiles derived from radioimmunoprecipitation analysis using [<sup>3</sup>H]glucosamine (Olmstead et al. (1989) *PNAS U.S.A.* 86:4355-4360 and Hosie et al. (1990) *AIDS* 4:215-220) or [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (O'Connor et al. (1989) *J. Clin. Micro.* 27:474-479), have demonstrated a gp100-120 band for envelope, a gp36-41 diffuse band for transmembrane, and a gp130-140 band for envelope precursor. The molecular weights of the viral protein components, as described by the above analyses, correspond to the immunoblot patterns of 24-28 kD for major core, 15-17 kD for minor core, 10 kD for minor core, 54-55 kD for core precursor, 62 kD for RT, 32 kD for endonuclease, 37-44 kD (diffuse band) for transmembrane, and 100-120 kD for envelope (Yamamoto et al (1988) *supra*; Hosie et al. (1990) *supra*; and O'Connor et al. (1989) *J. Clin. Micro.* 27:474-479). In this study, the development of antibodies to major core protein p28 was observed prior to the development of antibodies to the envelope glycoprotein gp100 in both immunization studies (A and B). Our immunoblot analysis of the sera from immunized cats closely resembled the immunoblot profiles of FIV-infected cats previously published by our laboratories and others (Yamamoto et al. (1988) *supra*; Hosie et al. (1990) *supra*; and O'Connor et al. (1989) *J. Clin. Micro.* 27:474-479). Comparison of high dose (200 g) indicates that large amounts of viral proteins are required to adequately and rapidly induce FIV antibodies (B). The immunoblot numbers represent the cat identification numbers.

FIGS. 6A and 6B: The FIV IgG antibody titer was measured by enzyme-linked immunosorbent assay (ELISA) using 250 ng/microwell of sucrose-gradient purified FIV as substrate and biotinylated goat anti-cat IgG (Vector Laboratories, BA-9000) as conjugating antibody (Pedersen et al (1987) *Science* 235:790-793). Sera from the different bleeding dates of each cat were serially diluted and assayed simultaneously in a single test. The results are based on two separate ELISA tests. Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIG. 7: Immunoblot analysis was performed on sera at a final dilution of 1:50 from cats immunized with fixed cell-virus or inactivated whole-virus vaccines. Results presented are those from cats immunized with fixed FIV-FL-4 cells (Group 1B), inactivated FIV

(Group 2A), or fixed uninfected FeT1 cells (Group 1C). Lane A is an immunoblot profile of a SPF cat experimentally infected with FIV.

FIGS. 8A and 8B: The neutralizing antibody titers to FIV were assayed the FIV-susceptible feline lymphoid cell line FeT1. In brief, diluted samples of heat-inactivated serum (56° C. for 30 min) were incubated with 100 tissue culture infective doses (TCID<sub>50</sub>) of FIV (Petaluma strain) for 45 min at 37° C. in a 25-cm flask. The FeT1 cells were added to this mixture at a final concentration of  $2 \times 10^5$  cells/ml. After three days of culturing, the cells were washed once with Hank's balanced salt solution to remove residual virus from the culture and then resuspended in fresh culture media (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 10 mM HEPES buffer, 50 µg/ml gentamicin, 1 × 10<sup>-5</sup>M 2-mercaptoethanol, and 100 U/ml human recombinant IL-2). Virus infection was monitored by Mg<sup>++</sup>-dependent RT assays of the culture fluid. The serum was considered positive for neutralizing antibodies when RT activity was  $\leq 50\%$  of the infected control culture which had no serum exposure. Nonspecific antiviral activity (i.e., interferon activity) was not detected in the heat-inactivated serum samples using the antiviral assay with vesicular stomatitis virus (Yamamoto et al (1986) *Vet. Immunol. Immunopathol.* 11:1-19). Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIGS. 9A and 9B: Cellular immunity of the cats immunized with the fixed cell-virus vaccine was monitored by assaying the PBLs for their ability to proliferate (FIG. 9A) or produce IL-2 (FIG. 9B) upon stimulation with inactivated whole FIV (1.25% paraformaldehyde inactivated). The PBLs were isolated from blood harvested at 27 weeks pc from all challenged animals and at 14 weeks post-immunization from unchallenged cats. The proliferation assay consisted of <sup>3</sup>H-thymidine incorporation by PBLs ( $1 \times 10^5$  cells/microwell) upon stimulation with inactivated FIV (4.5 µm/microwell) for five days at 37° C. Similarly, 1.5 ml-cultures of PBL ( $1 \times 10^6$  cells/ml) were incubated with FIV antigens (50 µm/ml) for two days and the culture fluid was assayed for IL-2 titer. The IL-2 assay consisted of measuring the amount of <sup>3</sup>H-thymidine incorporation of the IL-2-dependent murine HT-2C cells in presence or absence of IL-2 containing samples (Gillis et al. (1978) *J. Immunol.* 120:2027-2032). When compared to infected, unvaccinated control cats, the vaccine protected cats and the vaccinated but unchallenged cats responded significantly (stimulation index  $\geq 2.0$ ) to FIV antigenic stimulation in both proliferation ( $P < 0.001$ ) and IL-2 induction ( $P < 0.001$ ) assays. The P value was derived by using two-tailed t-test. Part A presents the results from the proliferation assay and part B the results from the IL-2 induction assay.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A novel virus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV) has been discovered and isolated in substantially pure form. The virus is infectious in cats, causing a wide variety of symptoms, including abortion, alopecia, anemia, chronic rhinitis, conjunctivitis, diarrhea, emaciation, enteritis, gingivitis, hematochezia, neurologic abnormalities, periodontitis, and seb-

orrheic dermititis. The course of the disease is usually fatal.

The etiology, pathogenesis, and morphology of FIV closely resemble those of human immunodeficiency virus (HIV) and simian T-lymphotropic virus III (SAIDS), which cause acquired immunodeficiency syndrome in humans and primates, respectively. FIV does not appear to be antigenically related to HIV or to SAIDS, but rather appears to be a species-adapted lentivirus that has existed in cats for some time. Preliminary surveys conducted by the inventors herein indicate that FIV infection in cats may be widespread, possibly accounting for a significant proportion of the immunodeficiency symptoms found in cats who are free from FIV infection.

FIV is a felid immunodeficiency virus characterized as a retrovirus, more specifically as a lentivirus, which is tropic for T-lymphocytes of the host which it infects. The virus is also characterized by horizontal transmission, and may further be characterized by vertical transmission in at least some cases.

It is expected that FIV is polymorphic, and reference to FIV in the present application is intended to encompass the entire FIV family, including a variety of strains which share substantial amino acid sequence and nucleotide sequence homology and which are immunologically related. Substantial amino acid sequence homology means at least about 75% homology, usually at least about 80% homology, and frequently 90% homology and above in at least some of the viral genes and proteins. For example, the env, gag, or pol regions may display the requisite homology, while the genome as a whole does not. In such cases, so long as the viruses are immunologically related, the viruses will be considered to be FIV within the ambit of the present invention.

By immunologically related it is meant that the various strains will display substantial serologic cross-reactivity with the newly-discovered strain which has been deposited. Serologic cross-reactivity is defined as the ability of an antiserum or antibodies specific for the deposited FIV strain to react with other FIV strains as well as the deposited strain. Usually, immunologically related strains will cross-react with antibodies specific for more than one epitopic site, usually more than five epitopic sites, and frequently ten or more epitopic sites.

Conveniently, FIV strains may be identified by Western blot analysis where purified virus is disrupted with a suitable detergent, e.g., sodium dodecyl sulfate, and separated on a slab gel by electrophoresis. The separated polypeptide bands are transferred from the gel to nitrocellulose filter paper and visualized with labelled antibody. The molecular weights of the various resolved bands may then be determined by comparison to known molecular weight standards. Substantial similarity between the Western blot analysis of an unidentified virus and that of a known FIV virus indicates that the unknown virus is likely an FIV virus.

Other FIV isolates have been characterized, indicating that the nucleotide sequence of the envelope gene varies by no more than about 15% among isolates. Such isolates, from different regions, are described in Masashi et al. (1990) In: Proc. 6th Intnl. Conf. AIDS, June 20-24, San Francisco, Abstract Th.A. 284 (Japanese isolate); Phillips et al. (1990) *J. Virol.* 64:4605-4613 (San Diego, California); Olmsted et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:2448-2452 (Petaluma, California); Talbot et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5743-5747 (Petaluma, California); Rigby et al. (1991)

In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, Cameron House, Loch Lomond, Scotland, May 28-31, page 42 (Scotland); and Siebelink et al. (1991) In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, supra. (The Netherlands). Any of these isolates could be used for preparing vaccines and cell lines according to the present invention.

FIV encodes an RNA-dependent DNA polymerase (reverse transcriptase) which is Mg.<sup>+2</sup>-dependent with maximal activity occurring at a Mg.<sup>+2</sup> concentration of approximately 5 mM and pH of approximately 7.8. FIV bands at a density of about 1.15 g/cm<sup>3</sup> in a continuous sucrose gradient. Western blotting of FIV-infected cell lysate yields major bands at approximately 22 to 28 kD, usually about 26 kD; 50 to 60 kD, usually about 55 kD; and 28 to 36 kD, usually about 32 kD.

FIV may be isolated from the sera of infected cats by conventional techniques. For example, peripheral blood lymphocytes (PBL) may be isolated from the blood of infected cats and placed in suitable culture media. The cultures are incubated, with normal PBL's being periodically introduced to the culture in order to maintain its viability as the original cells are killed by the virus. The infected cells should be placed in fresh culture medium periodically, and the virus may be recovered from the supernatant of the cell culture by sucrose-gradient separation, or other known separation techniques.

The FIV may also be obtained from other specimens, particularly from the lymph tissues of infected animals. The lymph tissues are broken and then suspended in culture medium, and the procedures described above are then carried out.

Compositions according to the present invention include the whole virus, as well as portions of the virus. The whole virus may be maintained in in vitro culture, as described above, or may be viably frozen at a temperature at or below about -78° C. (solid CO<sub>2</sub>-dry ice), usually in the presence of agents which promote amorphous, vitreous solidification rather than crystallization. Suitable agents include glycerol and dimethylsulfoxide. Portions of the FIV of particular interest include the structural and regulatory proteins encoded by the FIV genome, including the envelope and core proteins, and fragments thereof.

The FIV may also be maintained in chronically infected cell lines, particularly T-cell lines, as described in detail in the Experimental section hereinafter. For example, interleukin 2 (IL-2)-dependent T-cell lines can be infected with FIV and maintained in IL-2-supplemented culture media. IL-2-independent cell lines can then be prepared by repeated subculturing with a gradual depletion of IL-2. Surviving cultures can then be maintained in culture free from IL-2. The IL-2-independent FIV-infected cell lines have been found to possess enhanced viability and a reduced percentage of syncytial cells when compared to IL-2-dependent FIV-infected cell lines. See, Experimental section hereinafter.

The FIV used for infecting the cell lines may be isolated from infected cats, as described above, or may be obtained from the deposited Petaluma strain of the virus (A.T.C.C. VR 2186).

Particular non-infected feline T-lymphocyte cell lines (IL-2 dependent) which may be infected to produce chronically FIV-infected cell lines are designated FeT-1M (A.T.C.C. Accession No. CRL 10775) and FeT-2D

(A.T.C.C. Accession No. CRL 10774), both deposited at the American Type Culture Collection, Rockville, Maryland, on Jun. 7, 1991.

Particular FIV-infected cell lines (IL-2 independent) which have been established from FeT-1M are FL-4 (A.T.C.C. Accession No. CRL 10772) and FL-6 (A.T.C.C. Accession No. CRL 10773), both deposited at the American Type Culture Collection on Jun. 7, 1991. Both these cell lines have been found to be prolific producers of FIV.

FeT-1M, FeT-2D, FL-4 and FL-6 were developed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

Polypeptides of the present invention will be either haptenic or antigenic, including at least six amino acids, usually at least nine amino acids, and more usually twelve or more amino acids found contiguously within one of the natural FIV proteins. Polypeptides will generally correspond to at least one epitopic site which is characteristic of FIV. By characteristic, it is meant that the epitopic site will allow immunologic detection of the virus in a physiological sample with reasonable assurance. Usually, it will be desirable that the epitopic site be immunologically distinct from (i.e., not cross-reactive with antibodies which recognize) viruses other than FIV. In some cases, however, it may be desirable that the epitopic site be immunologically similar to other viruses.

The FIV polypeptides may be natural, i.e., including the entire FIV protein or fragments thereof isolated from a natural source, or may be synthetic. The natural polypeptides may be isolated from the whole virus which is obtained as described above by conventional techniques, such as affinity chromatography. Conveniently, polyclonal or monoclonal antibodies obtained according to the present invention (as described in more detail hereinbelow) may be used to prepare a suitable affinity column by well-known techniques. Such techniques are taught, for example, in Hudson and Hay, *Practical Immunology*, Blackwell Scientific Publications, Oxford, United Kingdom, 1980, Chapter 8.

Synthetic polypeptides which are immunologically cross-reactive with a natural FIV protein may be produced by either of two general approaches. First, polypeptides having fewer than about 100 amino acids, more usually fewer than about 80 amino acids, and typically fewer than about 50 amino acids, may be synthesized by the well-known Merrifield solid-phase synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc., 85:2149-2156).

The second and preferred method for synthesizing the polypeptides of the present invention involves the expression in cultured cells of recombinant DNA molecules encoding a desired portion of the FIV genome. The portion of the FIV genome may itself be natural or synthetic, with natural genes obtainable from the isolated virus by conventional techniques. Of course, the genome of FIV is RNA, and it will be necessary to transcribe the natural RNA into DNA by conventional techniques employing reverse transcriptase. Alternatively, polynucleotides may be synthesized by well-known techniques. For example, short single-stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981), *Tetrahedron Letters* 22:1859-1862. Double-stranded fragments may then be obtained either by synthesizing the complementary strand and then annealing the

strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The natural or synthetic DNA fragments coding for the desired FIV protein or fragment may be incorporated in a DNA construct capable of introduction to and expression in *n* *vitro* cell culture. Usually, the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria. They may also be intended for introduction and integration within the genome of cultured mammalian or other eukaryotic cells. DNA constructs prepared for introduction into bacteria or yeast will include a replication system recognized by the host, the FIV DNA fragment encoding the desired polypeptide product, transcriptional and translational initiation regulatory sequences joined to the 5'-end of the FIV DNA fragment, and transcriptional and translational termination regulatory sequences joined to the 3'-end of the fragment. The transcriptional regulatory sequences will include a heterologous promoter which is recognized by the host. Conveniently, a variety of suitable expression vectors are commercially available for a number of hosts.

To be useful in the detection methods of the present invention, the polypeptides are obtained in a substantially pure form, that is, typically from about 50% W/W or more purity, substantially free of interfering proteins and contaminants. Preferably, the FIV polypeptides are isolated or synthesized in a purity of at least 80% W/W, and more preferably, in at least about 95% W/W purity. Using conventional protein purification techniques, homogeneous polypeptide compositions of at least about 99% W/W purity can be obtained. For example, the proteins may be purified by use of the antibodies described hereinafter using the immunoabsorbant affinity columns described hereinabove.

Once a sufficient quantity of natural or synthetic FIV polypeptides have been obtained, polyclonal antibodies specific for FIV may be produced by *n* *vitro* or *in vivo* techniques. *In vitro* techniques involved *in vitro* exposure lymphocytes to the antigenic polypeptides, while *in vivo* techniques require the injection of the polypeptides into a wide variety of vertebrates. Suitable vertebrates are non-human, including mice, rats, rabbits, sheep, goats, and the like. Polypeptides having more than about thirty amino acids, usually more than about fifty amino acids, may serve directly as the immunogen. If the polypeptide is smaller than about 10kD, particularly less than about 6kD, however, it may be necessary to join the polypeptide to a larger molecule to elicit the desired immune response. The immunogens are then injected into the animal according to a predetermined schedule, and the animals are bled periodically with successive bleeds having improved titer and specificity. Injections may be made intramuscularly, subcutaneously, or the like, and an adjuvant, such as a combination of complete and incomplete Freund's adjuvant, will usually be employed. The whole virus can also be used as the immunogen, although selection of antibodies specific for a particular determinant will be more difficult.

If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having the desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse, is hyperimmunized with the desired antigen by the method just described. The vertebrate is then killed,

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usually several days after the final immunization, the spleen removed, and the spleen cells immortalized. The manner of immortalization is not critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1976) *Eur. J. Immunol.* 6:511-519. Other techniques include EBV transformation, transformation with oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with a fusion partner, the manner of fusion is not critical and various techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of a non-ionic detergent, usually polyethylene glycol, and other additives such as Dulbecco's Modified Eagle's medium, for a few minutes. At the end of the fusion, the non-ionic detergent is rapidly removed by washing the cells. The fused cells are promptly dispensed in small culture wells (usually in a microtiter plate at relatively low density, ranging from about one to  $5 \times 10^5$  cells/well), in a selective medium chosen to support growth of the hybrid cells while being lethal to the myeloma cells. Usually, the myeloma cell line has been mutated to be sensitive to a lethal agent, typically being HAT sensitive, and the medium includes a HAT concentration sufficient to inhibit the proliferation of the unfused myeloma cells.

After sufficient time, usually from about one to two weeks, colonies of hybrids are observed and plates containing hyperpositive wells are identified. The plates and wells having only one colony per well are selected, and supernatants from these wells are tested for binding activity against FIV or a particular FIV protein. Once positive hybridomas are identified, the cell line can be maintained as a viable culture and/or a quantity of the virus may be grown out, separated, and stored by lyophilization.

Depending on the desired use for the antibodies, further screening of the hybridomas may be desirable. For use in immunodiagnostic assays, antibodies having very high specificity and affinity for the antigenic site are desirable.

Once the desired hybridomas have been selected, monoclonal antibodies may be isolated from supernatants of the growing colonies. The yield of antibodies obtained however, is usually low. The yield may be enhanced by various techniques, such as injection of the hybridoma cell line into the peritoneal cavity of a vertebrate host. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Proteinaceous and other contaminants will usually be removed from the monoclonal antibodies prior to use by conventional techniques, e.g., chromatography, gel filtration, precipitation, extraction, or the like.

The polypeptides and antibodies of the present invention may be used with or without modification for the detection of or vaccination against FIV infection. Frequently, the polypeptides and antibodies will be labelled by joining, either covalently or non-covalently, a substance which provides for detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Some of the labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescers, chemiluminescers, magnetic particles and the like. Patents teaching the use of such labels include U.S.

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Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies and polypeptides prepared as described above can be used in various immunological techniques for detecting FIV and anti-FIV antibodies in physiological specimens, particularly body fluid samples, including blood, plasma, serum, urine, and the like, and cell samples, such as lymphocytes. Depending on the nature of the sample, both immunoassays and immunohistochemical staining techniques may find use.

Liquid phase immunoassays and Western blot analysis will find use in detection of FIV in body fluids, particularly blood and urine. The use of antibodies in protein binding assays is well established. Numerous competitive and noncompetitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available. Detailed methods for detecting the presence of the viruses in serum samples are set forth in the Experimental section hereinafter. Additionally, enzyme linked immunosorbent assays (ELISA) for detecting presence of antibodies to FIV in blood are also set forth in the Experimental section.

Compositions of the present invention are also useful in preparing vaccines for protection against FIV infection. For example, the whole virus and/or FIV-infected cell lines may be wholly or partially inactivated and utilized as an immunogen in a vaccine composition. Partial inactivation may be achieved by passage at elevated temperatures or by contact with mutagens, such as ultraviolet light, ethyl methanesulfonate, and the like. Complete inactivation may be achieved by contact with other agents, including formalin, paraformaldehyde, phenol,  $\alpha$ -lactopropionate, ultraviolet light, heat, psorlens, platinum complexes, ozone and other viricidal agents.

Specific methods for the preparation of inactivated whole virus and FIV-infected cell line vaccines are described in detail in the Experimental section hereinafter. Conveniently, the source of whole FIV can be FIV-infected cell lines which have been found to be prolific producers, such as FL-4 and FL-6. Inactivated FL-4 and FL-6 are also suitable for preparing inactivated or attenuated whole cell vaccines.

The viral proteins and portions thereof, prepared as described above, may also be used in the preparation of subunit vaccines prepared by known techniques. Polypeptides displaying antigenic regions capable of eliciting protective immune response are selected and incorporated in an appropriate carrier. Alternatively, an antigenic portion of a viral protein or proteins may be incorporated into a larger protein by expression of fused proteins. The preparation of subunit vaccines for other viruses is described in various references, including Lerner et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:3403 and Bhattacharjee et al. (1982) Proc. Natl. Acad. Sci. U.S.A. 79:4400. See also, U.S. Pat. Nos. 4,565,697 (where a naturally-derived viral protein is incorporated into a vaccine composition); 4,528,217 and 4,575,495 (where synthetic peptides forming a portion of a viral protein are incorporated into a vaccine composition). Other methods for forming vaccines employing only a portion of the viral proteins are described in U.S. Pat. Nos. 4,552,757; 4,552,758; and 4,593,002. The relevant portions of each of these cited references and patents are incorporated herein by reference.

The vaccines prepared as described above may be administered in any conventional manner, including

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oronasally, subcutaneously, intraperitoneally or intramuscularly, except that oronasal administration will usually not be employed with a partially inactivated virus vaccine. Adjuvants will also find use with subcutaneous and intramuscular injection of completely inactivated vaccines to enhance the immune response. The preparation of viral vaccine compositions optionally employing adjuvants is described in numerous standard references, such as *Remington's Pharmaceutical Sciences*, 10 Mack Publishing Co., Easton, Pa., 16th ed., 1982, the disclosure of which is incorporated herein by reference.

The dosage form and immunogen content of the vaccine will vary depending on the nature of the immunogen (i.e., whole virus, infected cell, or subunit) and the route of administration. Usually, a single dose will have a total volume including carrier, adjuvant, and any other components, in the range from about 0.1 ml to about 5 ml, more usually being from about 0.5 ml, more usually being from about 0.5 ml to about 3 ml. The amount of inactivated or attenuated whole FIV in each dose will usually be in the range from about 0.1 mg to about 5 mg, usually being from about 0.2 mg to 2 mg. For inactivated FIV-infected cell lines, each dose will typically contain from about  $10^6$  to  $10^8$  cells, usually about  $5 \times 10^6$  to  $5 \times 10^7$  cells.

The number and temporal spacings of the inoculations will be sufficient to elicit the desired immunoprotective response against subsequent challenge by FIV. Usually, there will be at least two inoculations spaced at least one week apart, more usually being from two to 10 inoculations spaced over a period from two to thirty weeks. Often, a final inoculation may be administered at some longer interval following an initial series of administrations. The selection of optimum administration patterns for a particular vaccine formulation is well within the skill in the art.

Diagnostic tests for detecting the presence of FIV in biological samples may also be performed using polynucleotide probes. Such polynucleotide probes may be prepared based on the sequence of the viral genome. The length of the probe is not critical, but will usually comprise at least about 12 bases, more usually comprising at least about 16 bases, which are substantially complementary to a portion of the viral genome. The probe itself may be DNA or RNA, and the probe need not have perfect complementarity with the FIV genome, with one or two mismatched pairs being acceptable for probes up to 20 bases in length and three to five mismatched pairs in probes from 20 to 35 bases. The probes may be prepared synthetically, with suitable synthetic techniques having been described above, and will include a detectable label. Usually, the synthetic sequences are expanded in commonly available cloning vectors and suitable hosts in order to obtain large quantities. The expanded vectors may themselves be labelled for use as probes, or shorter fragments containing complementary strands may be excised and labelled. Methods for the preparation and utilization of nucleotide probes for diagnostic testing are described in Falkow et al. U.S. Pat. No. 4,358,535, the disclosure of which is incorporated herein by reference.

A variety of labels have been employed, including those which have been described above for use in immunoassays, particularly radionuclides. Suitable labels may be bound to the probe by a variety of techniques. Commonly employed is nick translation with  $\alpha$ - $^{32}$ P-dNTP terminal phosphate hydrolysis with alkaline phosphatase followed by 5'-end labelling with radioac-

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tive<sup>32</sup>P employing 7-P-NTP and T4 polynucleotide kinase or 3'-end labelling with an  $\alpha$ -<sup>32</sup>P-dNPT and terminal deoxynuclotidyl transferase. Alternatively, nucleotides can be synthesized where one or more of the atoms present are replaced with a radioactive isotope, e.g., hydrogen with tritium. In addition, various linking groups can be employed. The terminal hydroxol can be esterified with inorganic acids, e.g., <sup>32</sup>P phosphate or <sup>14</sup>C organic acids, or else esterified with bifunctional reagents to provide other reactive groups to which labels can be linked.

The following examples are offered by way of illustration, not by way of limitation.

The experimental work described below relating to the development and use of cell lines FeT-1M, FeT-2D, FL-4, and FL-6 was performed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

## EXPERIMENTAL

### Materials and Methods

#### Cell Types

Cells used as the source of FIV were the Crandell feline kidney cell line (FIV-CRFK) and feline mixed fresh PBLs (FIV-FeT1). Both cell types were infected with the Petaluma strain of FIV (A.T.C.C. No. VR 2186; deposited on Aug. 5, 1987, in connection with parent application Ser. No. 07/089,700). The FIV-CRFK line grows as a monolayer, morphologically similar to uninfected CRFK cells (Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258 and Fabricant et al. (1971) J. Am. Vet. Med. Assoc. 158:976-980). FIV-FeT1 cells, like uninfected FeT1 cells (mixed peripheral blood lymphocyte (PBL) cells from specific pathogen free (SPF) cats), grow in suspension and require interleukin-2 (IL-2). The IL-2-independent feline leukocyte cell lines, FL-4 and FL-6, were derived from the FIV-FeT1 cells and also are suspension cells which spontaneously produce FIV.

#### Cell Cultures

All suspension cell lines used in this study (FeT1, FL-4, FL-6) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES (N-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid), 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$ M 2-mercaptoethanol. IL-2-dependent cells were supplemented with 100 U/ml of recombinant human IL-2 (Cetus Corporation, Emeryville, Calif.). The suspension cells were passaged at a cell concentration of  $0.5-4 \times 10^6$  cells/ml and recultured in fresh culture media twice a week. FIV-CRFK cells were cultured in media consisting of equal volumes of L-15 and Eagle's minimum essential media, 10% heat-inactivated FCS, and 50  $\mu$ g/ml gentamicin. All monolayer cells were passaged twice a week at an initial cell concentration of  $2 \times 10^6$  cells/ml. The FIV-infected tissue culture fluids (TCF) were harvested twice a week, spun at 3000 rpm for 1 hr to remove residual cells, and stored at -20° C. or -70° C. or at 5° C. for those scheduled to be used within 1-5 days. One ml samples of cell-free infected TCF were routinely tested for Mg<sup>++</sup>-dependent reverse transcriptase (RT) activity as a means of monitoring for FIV production. Infected TCF were also checked routinely for Mn<sup>++</sup>-dependent RT activity to ensure that the cultures were producing only Mg<sup>++</sup>-dependent feline retrovirus (i.e., FIV). The RT assay used poly(rA)oligo(dT<sub>12-18</sub>) as an exogenous tem-

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plate primer, four different deoxyribonucleotide triphosphates, 20 mM KCl with Mg<sup>++</sup> for detecting FIV or 60 mM NaCl with Mn<sup>++</sup> for detecting Mn<sup>++</sup>-dependent viruses (such as FeLV) and 5  $\mu$ Ci (<sup>3</sup>H)TTP alone per sample (Rey et al. (1984) Biophys. Res. Commun. 121:126-133). Five  $\mu$ Ci of (<sup>3</sup>H)TTP gave an average total count of 450,000 cpm using scintillation fluid mixture (1 part xylene to 2 part Amersham biodegradable counting scintillant) on a Beckman LS250 scintillation counter. As a result, our RT values will be below 450,000 cpm/ml.

#### Development of IL-2-Independent FIV Producer Lines

IL-2-independent FIV producing cell lines were developed from an IL-2-dependent FIV-infected feline PBL line (FIV-FeT1). The process of gradual IL-2 depletion from the FIV-FeT1 cell line took extensive sub-culturing over a period of approximately three months. The depletion process entailed a gradual reduction of the percentage of IL-2 containing media from the culture in the following weekly sequence: 75%, 50%, 25%, 5% and 0% IL-2-containing media. During this period over 80% of the starting cultures which were depleted of IL-2 did not survive the procedure. Surviving cultures were placed in individual 2-cm<sup>2</sup> multiwells at a viable cell concentration of  $2 \times 10^6$  cells/ml/well. During this stage only three of starting 20 cultures survived and these cultures were expanded sequentially into 25-cm<sup>2</sup>, 75-cm<sup>2</sup>, and 175-cm<sup>2</sup> flasks. One of the cultures (FL-5) did not survive. RT assays were performed on the surviving two cultures (FL-4 and FL-6) during the expansion period as means of monitoring FIV production.

#### In Vitro Infectivity Studies

The clarified infectious TCF from FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells was filtered individually with 0.45  $\mu$ m sterile filters to remove residual cells. These FIV inocula were aliquoted into 8-ml samples, stored at -70° C. and samples of these frozen inocula were retested for RT activity prior to in vitro infectivity studies. In all studies, the frozen inocula were thawed at room temperature immediately prior to use. FIV-susceptible feline cells ( $1 \times 10^6$  cells/ml) were infected with FIV at RT activity of 30,000 cpm/ml. All of the FIV-susceptible cells used in this study, with the exception of uninfected CRFK, were IL-2-dependent lymphoid cells which grew in suspension and required no trypsinization for passage. The TCF of the infected test cultures was harvested twice a week and the cells were recultured in fresh culture media containing IL-2. The harvested TCF was routinely tested for RT activity.

#### In Vivo Infectivity Studies

Two specific pathogen free (SPF) cats, 11 months of age, were inoculated intraperitoneally (IP) with 2 ml of infectious TCF from either FL-4 or FL-6 cells. Infectious TCF from FL-4 or FL-6 cultures, having RT activities of 150,000 cpm/ml, was aliquoted and stored at -70° C. The frozen virus inocula were thawed at room temperature and filtered with 0.45  $\mu$ m Millipore filter just prior to the inoculation. Both the single freeze-thawing and the filtering procedure ensured that the inocula were free of viable cells. The cats were bled routinely to obtain serum for serological assays and PBLs for virus isolation. Virus isolation consisted of

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co-cultivating  $2-10 \times 10^5$  cells/ml with equal number of FIV-susceptible uninfected FeT1 cells and monitoring the TCF from these cultures for six weeks by RT assay. The PBL were considered positive for FIV isolation when RT activity of  $> 10,000$  cpm/ml were detected in TCF from at least two consecutive harvest days. The RT activity of the TCF from co-culturing PBL from SPF cats with FeT1 cells was  $< 2,500$  cpm/ml.

#### FIV Purification

FIV from infected TCF was concentrated and purified by ultracentrifugation, first on a 10/50% (w/v) discontinuous sucrose gradient and then on a 10/50% continuous sucrose gradient (Pedersen et al. (1987) Science 235:790-793 and Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S). The virus purified by this procedure was used for comparing the biochemical properties of FIV derived from different culture preparations and as the viral substrate for the immunoblot assay. Immunoblot analyses of gradient purified FIV from different infected cell lines (FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells) demonstrated the presence of the envelope gp100 band in blots from all viral sources. However, one major difference observed during these studies was that the intensity of the gp100 band was always weaker on the immunoblots made from purified FIV of FIV-CRFK origin than from those produced by other infected cell lines. Consequently, more viral antigen from FIV-CRFK cells was needed on the blots to get comparable intensity at the envelope band.

#### Immunoblot Analysis

A modification of the immunoblot technique described by Carlson et al. was used (Carlson et al. (1985) JAMA 253:3405-3408). Serum samples from immunized or FIV-infected cats were diluted to 1:50 in Buffer 3 (0.15M sodium chloride, 0.001M ethylene diaminetetraacetic acid, 0.05M Tris base, 0.05% Tween 20, and 0.1% bovine serum albumin) and incubated with the virus blot strips in individual wells for 18 hours at 37° C. These blot strips were then processed using a modification of a previously described procedure (Yamamoto et al. (1988) supra.). Briefly, the strips were incubated individually in wells with biotinylated anti-cat IgG (Vector Laboratories, Burlingame, Calif.) for 30 min and washed three times with wash solution. The strips were then incubated individually with horseradish peroxidase Avidin D (Vector Laboratories) for 30 min. After extensive washing, the strips were incubated with a fresh substrate solution (0.05% diaminobenzidine, 400 µg/ml NiCl<sub>2</sub> and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.1M Tris buffer, pH 7.4) at room temperature. After establishment of visible bands the reactions were stopped with excess distilled H<sub>2</sub>O, and the strips were then dried.

#### FIV p28 Assay

The FIV core protein p28 was detected by an enzyme-linked immunoassorbent assay (ELISA) using two different monoclonal antibodies. A1 and B1 mAbs, to FIV p28 as either capture or substrate-reactive antibodies, respectively. Reactivity of both mAbs to FIV p28 antigen was confirmed by immunoblot analysis. The capture antibody (mAb A1) was coated on the plate overnight with bicarbonate buffer (pH 9.6) and washed once before its use. Serum samples to be tested were diluted in Buffer 3 and then incubated in the coated wells for 30 min at 37° C. The wells were

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washed six times with washing buffer, incubated with biotinylated mAb B1 for 30 min at 37° C., washed six times more, and then incubated with horseradish peroxidase Avidin D for 15 min. The wells were washed extensively again and finally incubated with substrate solution (0.005% tetramethylbenzidine and 0.015% H<sub>2</sub>O<sub>2</sub> in 0.96% citric acid solution) at room temperature. The reactions were stopped with 1M sulfuric acid solution upon establishment of a visible color reaction in the sequentially diluted standards consisting of purified FIV from pooled FIV-CRFK and FIV-FeT1 preparations.

#### Characterization of FL-4 and FL-6 Cell Lines

15 The phenotypic profiles of the feline cells were determined by fluorescence activated cell sorter (FACS) analysis using characterized monoclonal antibodies to feline CD4 (FeL 7), CD8 (FT2), pan T-cell, and to feline light chain and  $\mu$  heavy chain specific (AC5) markers 20 (Ackley et al. (1990) supra.; Ackley et al. (1990) supra.; Klotz et al. (1986) J. Immunol. 136:2510-2516; and Klotz et al. (1985) J. Immunol. 134:95-99). The cells were tested for mycoplasma using two different procedures both performed by Bionique Laboratories, Inc. 25 The first procedure consisted of the direct DNA-/fluorochrome staining of the cells for mycoplasma. The second procedure involved passaging test cells onto indicator cells which were then DNA/fluorochrome stained for mycoplasma. Detection of FeLV p27 core 30 antigen was performed using the p27 antigen ELISA assay (Lutz et al. (1983) J. Immunol. Methods 56:209-220). Polymerase chain reaction (PCR) was used to test for the presence of FeLV provirus DNA. Briefly, a pair of primer sequences from the U3 region 35 of the FeLV LTR were chosen so as to avoid the possibility of overlap with endogenous sequences of FeLV. The sequences of the two oligonucleotides primers used for PCR were 14 base pairs (bp 24 to 37) and 17 base pairs (bp 239 to 255) long. This enabled us to amplify a 40 sequence of 232 base pairs for which we prepared a 25 base pair probe (bp 203 to 227) labeled with <sup>32</sup>P for identification by Southern blotting. The indirect fluorescent antibody assay to detect feline syncytium-forming virus (FeSFV) was performed as described previously (Pedersen et al. (1987) supra.; Yamamoto et al. (1988) supra.; and Yamamoto et al. (1989) J. Am. Vet. Med. Assoc. 194:213-220).

#### Immunogenicity of FIV Produced from FL-4 and FL-6 Cells

50 Eighteen SPF cats, 4-6 months of age, were used in these studies. Some of these cats were previously exposed to feline herpes virus (FHV)(A.T.C.C. C-27 strain) and the cats were free of FHV symptoms two-weeks prior to and during immunization. Three of the 55 cats were immunized four times with 200 µg of inactivated FIV (inactivated whole virus) particles that were produced by pelleting cell-free TCF of FL-4 cells. An additional three cats were immunized eight times with 20 µm of inactivated whole virus. Seven cats were 60 immunized either four or six times with  $1 \times 10^7$  cells per dose of inactivated FL-6 or FL-4 cells (inactivated whole cell-virus), respectively. The pelleted virus and the infected cells were each inactivated with 1.25% paraformaldehyde, dialyzed against PBS, and then combined with adjuvant just prior to immunization. The adjuvants used were either threonyl muramyl dipeptide (MDP) (Byars et al. (1987) Vaccine 5:223-228)

or a combination of Freund's complete and incomplete adjuvants. Control cats were immunized with either uninfected FeT1 cells with adjuvant or diluent with adjuvant. All cats were immunized at two week intervals for a total of four or eight immunizations, unless stated otherwise.

## RESULTS

### Development of IL-2-Independent FIV-Producing Cell Lines

The development of IL-2-independent cell lines from FIV infected mixed PBLs (FIV-FeT1 cells) entailed the gradual depletion of IL-2 from the cultures. Only two out of 20 cultures, FL-4 and FL-6, survived the depletion process. Significant RT titers (100,000–400,000 cpm/ml), Mg<sup>++</sup> cation-dependent, were detected in these cultures during the expansion and large scale-production stage. Electron microscopy demonstrated numerous typical lentivirus particles in these cultures (data not shown).

The growth rates of these cell lines were compared to those of FIV-FeT1 and FIV-CRFK. The viable cell doubling time for FL-6 was found to be approximately 24 hours, whereas the doubling time for FL-6 was found to be approximately 24 hrs, whereas the doubling time for FL-4 was approximately 48 hrs. Both cell lines grew at an exponential rate. From a starting cell concentration of  $5 \times 10^5$  cells/ml, peak viable cell counts were observed after 3–4 days of culturing. Viability of the cells present in these cultures ranged from 70 to 90% over the four day culturing period. The number of syncytial cells in the FL-4 and FL-6 cultures was less than 0.1%. In comparison, the viability of FIV-FeT1 cells was only 55 to 65% after 1 day of culture, which may be attributed to dependence on IL-2. In our hands, a majority of the IL-2-dependent feline (FeT1, FeT2) and murine (HT-2C, CTLL-2) lymphoid cell lines have similar viability profiles. In order to evaluate the correlation between cell growth and virus production, samples from different harvest days were assayed for RT activity (FIG. 1). At a starting cell concentration of  $5 \times 10^5$  cells/ml, peak RT titers were observed on Day 4 of culture. Based on the number of cells present on Day 4, FL-4 cells produced the highest and FIV-CRFK the lowest RT activity.

### Characterization of the FL-4 and FL-6 Cell Lines

The phenotypic profiles of FL-4 and FL-6 cells were determined by flow cytometric analysis using monoclonal antibodies (mAb) to feline CD4 (fel 7), CD8 (FT2), pan T-cell (42) markers (Ackley et al. (1990) J. Virol. 64:5652–5655; Carlson et al. (1985) supra.; Ackley et al. (1990) supra.; and Klotz et al. (1986) supra.) and mAb that detect both feline immunoglobulin light chains and  $\mu$  heavy chain (AC5) (Klotz et al. (1985) supra.) (FIG. 2). The FACS profiles demonstrated that FL-4 cells were CD4 $\pm$ , CD8 $\pm$ , and Pan-T $+$  whereas FL-6 cells were CD4 $-$ , CD8 $\pm$ , and Pan-T $+$ . Both cell lines were negative for surface IgM and  $\lambda$  and  $\kappa$  light chains. It should be noted that both CD4 and CD8 antigens were lost in cultures maintained for several months. FL-4 and FL-6 cells were >95% positive by IFA for surface FIV antigen expression using polyclonal antibodies to FIV (Table 1). Additional tests were performed to ensure that these cells were free of known contaminants which could limit their use. The results are summarized in Table 1. The two cell lines were mycoplasma-free both by direct DNA/fluoro-

chrome stain and indirectly by passaging onto indicator cells prior to staining. Furthermore, FL-4 and FL-6 cells were shown to be negative for FeLV core protein p27 expression by ELISA and for exogenous FeLV DNA by PCR. The cells were determined to be negative by IFA for feline syncytial-forming virus (FeSFV).

TABLE 1

Absence of Known Contaminants in FL-4 and FL-6 Cell Lines			
Micro-organism Tested	Infection Status	Antigen Detected	Method of Testing
FIV	+	viral RT <sup>a</sup>	Mg <sup>++</sup> -dependent RT
	+	viral antigens <sup>ab</sup>	Immunoblot analysis with FIV-positive serum.
	+	mature virion	Electron microscopy.
	+	whole cell	IFA with FIV-positive serum (>95% positive).
FeLV	—	viral RT <sup>a</sup>	Mn <sup>++</sup> -dependent RT.
	—	viral core	ELISA.
	—	p27	
	—	mature virion proviral LTR sequence in cellular DNA	Electron microscopy. PCR and Southern blot.
FeSFV	—	mature virion	Electron microscopy.
	—	whole cell	IFA with FeSFV-positive serum.
Mycoplasma	—	whole cell	Direct DNA/fluorochrome staining for mycoplasma. Indirectly by staining indicator cells which were passaged with FL-4 and FL-6 cells.

<sup>a</sup>These tests were performed on the tissue culture fluid harvested from the FL-4 and FL-6 cell cultures.

<sup>b</sup>Purified virus was disrupted with 0.1% SDS prior to its use in immunoblot production, as described in Methods.

### FIV Production in FL-4 and FL-6 Cell Lines

The amount of FIV produced from the FL-4, FL-6, FIV-FeT1 and FIV-CRFK cell lines was determined by comparing the total protein and RT levels of FIV in different fractions from sucrose gradient preparations (data not shown). High titers of both RT activity and total protein were observed in FIV preparations from FL-4, FL-6, and FIV-FeT1 cells. The FIV-CRFK produced low titers of FIV as demonstrated by the low levels of both protein concentration and RT activity in the fractions. The three peak fractions of the gradient purified virus from each cell line were pooled and measured for total protein concentration, RT titer, and viral core protein (p28) concentration. The results demonstrated a direct correlation between the p28, RT and the total protein levels present in the purified virus preparations. The viral antigen profiles of the FIV produced by the various cell lines were also compared by immunoblot analysis. Different concentrations of purified virus from different cell sources were used as substrate antigen for immunoblot strips. These immunoblots were then reacted with a set concentration of FIV-seropositive cat sera and the banding patterns evaluated. The immunoblot profiles from FL-4 and FL-6 cells were similar to those of FIV-FeT1 and FIV-CRFK (data not shown). The intensity of the bands, especially the viral envelope and transmembrane glycoproteins, produced on immunoblots from FL-4, FL-6 and FIV-FeT1 viral

substrates was stronger than that from FIV-CRFK. In addition, immunoblots of significantly higher quality were produced from the larger quantity of purified virus obtained from the TCF of FL-4 and FL-6. Thus, these results further indicate that a larger amount of virus antigens was produced by the FL-4 and FL-6 cell lines than by the FIV-FeT1 and FIV-CRFK.

#### Characterization of FIV Produced From FL-4 and FL=6 Cells

The FIV produced from FL-4 and FL-6 cells was tested for its ability to infect FIV-susceptible cell lines (FIG. 3). Cell-free TCF from different infected cell lines was inoculated into various feline cell cultures at a set RT concentration of 30,000 cpm/ml. FIV from FIV-CRFK cells did not readily infect certain feline lymphoid cells, in particular thymus-derived cultures, as compared to the FIV from FL-4 and FIV-FeT1 cells. The FIV from FL-6 cells was also highly infectious to FIV-susceptible cell lines (data not shown). Next, the FIV preparations produced from FL-4 and FL-6 cells were tested for their ability to infect SPF cats (FIG. 4). One SPF cat each was inoculated IP with 2 ml of cell-free TCF from either FL-4 or FL-6 cells. Both cats developed antibodies to FIV within four weeks post-infection. By sixteen weeks post-infection, the immunoblot profiles of these sera demonstrated the presence of antibodies to the majority of viral core antigens, but not to the viral envelope or transmembrane glycoproteins. Both cats were positive for virus isolation from PBLs. These studies demonstrated that the virus preparations from the FL-4 and FL-6 cell lines were highly infectious in both *in vitro* and *in vivo* systems.

#### Immunogenicity of FIV Produced From FL-4 and FL-6 Cells

Immunization of four cats with the inactivated FL-4 cell preparations ( $1 \times 10^7$  cells) led to the production of FIV antibodies specific for the viral core protein p28 soon after the second immunization (FIG. 5A). Antibodies to other viral antigens were demonstrated only after the third or fourth immunization (FIG. 5A). Thus the development of the antibodies in immunized cats closely mimics the FIV antibody development in experimentally infected cats (Yamamoto et al. (1988) *supra*. and Hosie et al. (1990) *supra*.). Control cats immunized with uninfected FeT1 cell preparations did not develop viral antibodies over the duration of the six immunizations.

Six other cats were immunized with inactivated FL-4-produced virus (200 µg) or inactivated FL-6 cell ( $1 \times 10^7$  cells) preparations together with a combination of complete and incomplete Freund's adjuvant instead of MDP (FIG. 5B). Both inocula led to the production of antibodies specific to the viral p28 shortly after the second immunization. Two out of the three cats immunized with the inactivated virus preparation developed antibody responses to viral envelope, whereas all three cats immunized with the inactivated FL-6 cell preparation developed antibodies to the envelope shortly after the second immunization. When other cats were immunized with 20 µg of pelleted inactivated virus in MDP) per dose, two out of three cats developed antibodies to the viral envelope, but only after the sixth immunization (FIG. 5B). Furthermore,  $1 \times 10^7$  viable FL-4 or FL-6 cells released into the TCF approximately 10 µg equivalence of purified virus (by sucrose gradient method) or approximately 30 µg equivalence of crude pelleted

virus when at their peak production level (data not shown). These findings suggest that it is more practical to use inactivated whole infected cells as an immunogen than inactivated whole virus for development of antibodies to the viral envelope. No difference in antibody development to viral p28 was observed between the infected cell or cell-free virus immunogens. Thus, our studies demonstrate that cats immunized with inactivated whole FIV-infected cells generate higher FIV envelope antibody titers more rapidly and more consistently than those immunized with inactivated cell-free whole virus.

#### Vaccination with Inactivated Cell Lines and Post Vaccination Challenge

The fixed cell-virus vaccine consisted of FIVFeT1 and FIV-FL-4 inactivated with paraformaldehyde. In each culture (which was subsequently inactivated) essentially 100% of the cells were productively infected with FIV and  $5 \times 10^7$  cells were required to obtain  $100\mu$ g of total viral protein. Analysis of the FIV-infected cells in both T-cell lines by immunoblot using serum from an FIV immunized cat and by Coomassie stain, showed that the vaccine preparations contained the env, gag and pol virion proteins and their precursors as well as some regulatory proteins and cellular proteins (data not shown). The adjuvant used was threonylmuramyl dipeptide (MDP) (Syntex SAF-A).

The infected cells were inactivated with 1.25% paraformaldehyde for 24 hrs and washed three times with phosphate buffered saline (PBS). The vaccine consisted of  $1 \times 10^7$  inactivated FIV-FeT1 cells (Group 1A) or FIV-FL-4 cells (Group 1B) mixed with 250 µg of MDP. All cats in Group 1 were specific pathogen free (SPF) cats of 4-6 months of age, which were previously exposed to feline herpes virus (FHV C-27 strain) and were free of FHV symptoms two weeks prior to and during immunization. Ten control cats were immunized with either uninfected FeT1 cells with MDP (Group 1C) or MDP alone (Group 1D). All cats were challenged IP with 10 animal infectious doses (AID<sub>100</sub>) of homologous FIV petaluma strain two weeks after the final immunization. For the whole-virus vaccine, FIV was pelleted from the culture fluid of FIV-FL-4 cells, inactivated with 1.25% paraformaldehyde for 24 hours, and dialyzed extensively against PBS. Cats in Group 2A were SPF cats which were immunized with the inactivated FIV particles Group 2B cats received  $1 \times 10^7$  inactivated uninfected FeT1 cells mixed with the inactivated virus. Three additional SPF cats were immunized with either adjuvant (Cat #182) or diluent (Cats #55D and #55H). All cats were challenged with 10 ID FIV (homologous strain) two weeks after the final immunization. Virus was isolated from PBL and bone marrow cells by co-culturing with FIV-susceptible FeT1 cells. PCR analysis was performed using the method previously described (Pedersen et al. (1989) *J. Virol.* 64:598-606).

Nine cats in total were vaccinated subcutaneously (SC) with  $1 \times 10^7$  cells mixed with MDP (250 µg) five times at two week intervals and a final boost was given two months later. Five cats received the FIV-FeT1 cells (Group 1A) and four cats received the FIV-FL-4 cells (Group B). Ten control cats were inoculated with the uninfected allogeneic T-cells mixed with adjuvant, or adjuvant alone (Groups 1C and 1D, Table 2). Both fixed cell-virus vaccines induced significant levels of FIV antibodies (1:5,000 to 1:50,000) after the first-boost

as detected by whole-virus ELISA (FIG. 6A). By immunoblot, all nine vaccinated cats showed antibody to the viral core protein p24 after the first boost and antibody to other viral antigens, including the envelope gp100, after the third or fourth immunization (FIG. 7). Antibody response to the cellular components of the vaccine was weak as determined by immunoblot reactivity to the fixed uninfected cells. FIV-neutralizing antibody titers of 1:800 to 1:1200 were reached after the final immunization; these titers were equal to or slightly higher than the titers observed in unvaccinated cats experimentally infected with FIV (FIG. 8A). No infectious virus was detected in the vaccinated cats prior to challenge. Control cats remained free of antiviral antibodies and of infectious virus during the immunization schedule. The results are summarized in Table 2.

Virus was recovered persistently after 5 weeks pc from the PBLs of one vaccinated cat (#178) and after 21 weeks pc from another vaccinated cat (#138). The PBLs of both animals were PCR positive at 21 weeks pc at which time infectious virus was isolated from their bone marrow. These two persistently infected cats showed a sudden rise in antibodies by ELISA at the time virus was recovered, and the antibodies remained high thereafter (FIG. 6A). Also, by immunoblot, both core and envelope antibodies persisted longer in these cats than in the protected cats. These late breakthroughs of infectious virus in cats that had previously appeared protected indicate that only time can confirm the absence of latent virus in the protected vaccines. At 27 weeks pc, the seven vaccine protected cats showed FIV specific cell mediated response (CMR) as

TABLE 2

Isolation of FIV from Vaccinated Cats Before and After FIV-challenge							
GROUP 1A		GROUP 1B		GROUP IC		GROUP CODE	
GROUP ID	CAT #	GROUP 2A	GROUP 2B	GROUP 2C			
135,137,142, 150,209	145,224,138, 178	136,147,164, 214,227	175,215,270, 271,278	55B,55F,094	55C,55I,177	182,55D,55H	
				VACCINE TYPE			
FIV-FeT1 Cells	FIV-FL-4 Cells	FeT1 Cells (uninfected)	Placebo	Whole Virus	Whole Virus + FeT1 Cells (uninfected)	Placebo	
<b>FIV ISOLATION</b> (Positive Cat #) <u>From PBL</u>							
Pre-challenge	-	-	-	-	-	-	-
2 weeks post-challenge (pc)	-	-	-	ND	ND	ND	ND
3 weeks pc	-	-	147,214,227	175,270,278	-	-	-
5 weeks pc	209	178	147,214,227	175,270,278	ND	ND	ND
7 weeks pc	-	178	ALL	ALL	-	SSI	ALL
17 weeks pc	-	178	ALL	ALL	-	-	ALL
21 weeks pc	-	138,178	ALL	ALL	ND	ND	ND
26 weeks pc	-	138,178	ALL	ALL	ND	ND	ND
<u>From Bone Marrow</u>							
21 weeks pc	-	138,178	ALL	ALL	-	-	ALL
<u>PCR OF PBL</u>							
21 weeks pc	-	138,178	ALL	ALL	-	-	ALL
<b>TOTAL # INFECTED</b>	1/5	2/4	5/5	5/5	0/3	1/3	3/3
<b>TOTAL # CHALLENGED</b>	(Transient)	(Persistent)	(Persistent)	(Persistent)	(Transient)		

- Indicates negative result.

Number indicates positive result from a specific cat with corresponding identification number.

ALL indicates that all cats in the specific group are positive.

ND indicates not done.

Two weeks after the final immunization, all of the cats were challenged intraperitoneally (IP) with 10 animal infectious doses (AID<sub>100</sub>) of the homologous FIV strain. Starting at seven weeks post challenge (pc) all ten control cats seroconverted, antibody titers gradually increased, and virus was persistently isolated from their peripheral blood lymphocytes (PBLs) (Table 2). By contrast, a steady fall in antibodies occurred and virus could not be isolated from the PBLs of six of the nine vaccinated cats for  $\geq 21$  weeks pc. These six cats were also free of detectable virus at 17 weeks pc as measured by bone marrow culture and polymerase chain reaction (PCR) analysis of PBLs and bone marrow cells. They therefore seemed to be solidly protected without evidence of latent proviral DNA. In one of the vaccinated cats (#209), virus was recovered from PBLs only one occasion, at 5 weeks pc, after which it was no longer detectable in either the PBLs (by virus isolation and PCR) or the bone marrow cells (by virus isolation). Antibody levels decreased steadily in this animal. Therefore, this animal may also be protected.

measured by positive lymphocyte proliferation and IL-2 induction assays (FIGS. 9A and 9B) as well as a positive response to non-specific mitogens (data not shown). By contrast, the two persistently infected vaccines and all infected control cats showed a lack of cellular response to FIV while the non-specific mitogen response remained intact. Since these cats were not tested for CMR before challenge we do not know if they were genetically poor responders and therefore vulnerable to infection or whether these defects in CMI were the result of infection. These findings suggest that the fixed cell-virus vaccine had induced T-cell immunity in the apparently protected cats. The duration of memory T-cell and the possible vaccine induction of cytotoxic T-lymphocytes remain to be determined.

#### Vaccination with Inactivated Whole Virus and Post Vaccination Challenge

The cell-free whole virus vaccine was prepared from FIV-FL-4. Virus released from this cell line in high titer

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( $5 \times 10^8$  cells produced 1 mg viral protein per litter) was pelleted, filtered (0.45  $\mu\text{m}$ ), inactivated with paraformaldehyde, and given with a combination of Freund's complete and incomplete adjuvants. Analysis of the cell-free pelleted FIV preparation from the whole-virus vaccine by immunoblot using serum from an FIV immunized cat showed that this vaccine contained all of the viral antigens, although a lesser amount of env glycoproteins than was present in the fixed infected cell vaccine, and also a trace amount of cellular antigens (data not shown). Six cats were immunized SC with 200  $\mu\text{g}$  viral proteins given every two weeks  $\times 4$ . Three control cats received either adjuvant or diluent alone. Significant levels of FIV antibodies (1:20,000 to 1:35,000) detected by whole-virus ELISA were induced after the first boost (FIG. 6B). By immunoblot, core p24 antibodies developed in all cats after the first boost and envelope gp100 antibodies developed in five of six cats after the third immunization (FIG. 7). Just prior to challenge, neutralizing antibody titers to FIV of 1:100 to 1:600 were present in all vaccinees (FIG. 8B). All cats were free of infectious virus prior to challenge.

Two weeks after the final immunization, cats were challenged IP with 10 ID of FIV. PBL cultures became virus and PCR positive by seven weeks pc from the three controls (Table 2), whereas five of six vaccinated cats remained uninfected up to 14 weeks. The PBLs of one vaccinated cat (#55I) were transiently infected at 7 weeks pc but were negative by virus isolation and PCR at 17 weeks pc. After challenge, gradual decreases in antibody titers were observed in all immunized and protected cats including the single transiently infected cat. These findings show that effective protection against FIV challenge infection can also be achieved with an inactivated whole-virus vaccine.

Both the fixed cell-virus and whole-virus vaccines appeared to give roughly equal protection against challenge infection. However, the whole-virus vaccine was less immunogenic and required about 20-fold more cells to produce the amount of viral protein required to raise antibody titers to the levels approximating those observed with the fixed cell-virus vaccine. Nevertheless, the neutralization titers were still several fold less with the cell-free virus vaccine. This difference can probably be attributed to the difference in adjuvant used and/or to the greater amount and integrity of viral antigens presented on infected cells as compared to cell-free virus (Yamamoto et al. (in the press) Intervirol., and Hosie and Jarrett (1990) AIDS 4:215-220). The cell-

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virus vaccine may also have elicited an allogenic effect from the inclusion of other cellular antigens. However, a mixture of uninfected allogeneic (FeT1) cells and inactivated whole virus (Table 2, Group 2B) did not enhance the ELISA and neutralizing antibodies to FIV as compared to whole virus alone. This indicates that the expression of viral antigens on the infected cell apparently provides the most effective immunogenicity.

The specific viral proteins and specific immune responses that account for the vaccine protection observed are as yet uncertain. Viral envelope appears an essential determinant because, in another trial, cats immunized with an FIV Iscom vaccine that was deficient in envelope antigen failed to make gp120 antibody and were not protected against challenge infection with 20 ID<sub>50</sub> of homologous virus. The vaccines of the present invention probably achieved a minimal threshold of protection because, using a similar fixed cell-virus vaccine we were previously unable to protect against a higher challenge dose ( $5 \times 10^3$  ID) of virus (data not shown). Although neutralizing antibody would seem a logical mechanism, other means of vaccine protection, such as antibody dependent complement lysis or cellular cytotoxicity (ADCC) against cell-free virus or infected cells, may also contribute.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A vaccine against feline immunodeficiency virus infection comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, wherein said immunogen elicits an immune response protective against infection by FIV when administered to a susceptible host in an amount effective to elicit such response.
2. A method for protecting a susceptible cat against feline immunodeficiency virus infection, said method comprising administering to said cat a vaccine comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, in an amount effective to elicit an immune response protective against infection by FIV.
3. A vaccine composition as in claim 1, further comprising an adjuvant.

\* \* \* \* \*

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ATTACHMENT D

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Page**Maintenance Fee Statement**

5275813

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 11, "STAT" below.

If a maintenance fee payment is defective, the reason is indicated by code in column 11, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,275,813	184	1950		07/739,014	01/04/94	07/31/91	08	NO	PAID

ITEM NBR	ATTY DKT NUMBER
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1 2307U-237-3/

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**BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV  
(1A55.20)**

Date	Description
28-Aug-91	Submitted FIV KV license application.
28-Aug-91	Submitted new FIV KV Production Outline.
28-Aug-91	Submitted research report entitled "Feline Immunodeficiency Virus Vaccine: Immunogenicity Study Proposal for USDA Licensure".
15-Nov-91	USDA approved the Production Outline with comments & pen-and-ink changes.
3-Feb-92	USDA responded to research report/protocol stating they are not yet prepared to state that the proposed study can meet the intended goal.
15-Jun-99	Requested USDA to transfer this product to their inactive files as we are discontinuing our efforts to license the product. The 2 strain product had, to this point, contained a strain (FIV alternate subtype A infected cell line) which we decided in 1999 to replace with the present strain (FIV subtype D infected cell line). The FIV subtype A infected cell line strain in the discontinued project remains in the present product due to be licensed (15A5.21). Although the 1A55.20 licensing project was cancelled in 1999, its FIV subtype A infected cell line fraction is actually the basis on which our 2001 license for 15A5.21 will be built.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
4-May-98	Submitted FIV-KV license application.
1-May-98	Submitted new FIV-KV Production Outline.
4-May-98	Submitted FIV-KV subtype A infected cell line master cell stock qualification report (MCS & MCS+25) and 2008s. Requested TA# to submit to CVB-L and permission to transfer to production.
4-May-98	Submitted FIV-KV alternate subtype A cell line master cell stock qualification report (MCS & MCS+25). Requested TA# to submit to CVB-L and permission to transfer to Production.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the subtype A infected cell line to Production at our own risk.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the alternate subtype A cell line MCS to Production at our own risk.
13-Aug-98	Submitted efficacy protocol.
5-Nov-98	The license application has been filed.
1-Dec-98	The new Outline was approved w/ pen-and-ink changes & comments.
	Letter (faxed) requesting permission to move virus fluid from R & D building to production room 520, bldg 115 to be killed by autoclaving.
9-Jun-99	Permission granted to move the virus fluid from R&D to Production. However, questions were raised with other CVB-IC personnel
2-Jul-99	Submitted letter for master seed/master cell stock qualification report for FIV subtype D infected cell line. Requested permission to submit cell line to CVB-L and transfer to our production department. Requested TA #.
15-Jul-99	7/15/99 received verbal permission to transfer FIV subtype D infected cell line to Production at our own risk & TA#7610 to submit MCS samples to CVB-L. Confirmation letter received on 7/23/99.
20-Jul-99	Submitted FIV subtype D infected cell line along with the X+25 to CVB-L for confirmatory testing.
20-Jul-99	Submitted subtype A infected cell line to CVB-L for confirmatory testing.
4-Aug-99	Responded to Renee's June 9, 1999, questions (approval was granted) in regard to our moving a bioreactor to Room 510 Building 115 for the purpose of autoclaving.
4-Aug-99	No response required to the questions in regard to moving a bioreactor to Room 510.
27-Aug-99	Submitted subtype A infected cell line samples for testing.
4-Oct-99	A revised efficacy protocol which includes sufficient detail to address USDA comments needs to be submitted for consideration by the CRT prior to initiation of the study.
12-Oct-99	Submitted a revised efficacy protocol in response to USDA's 10/4/99 letter prior to initiation of the study.
29-Dec-99	Approved the revised efficacy protocol for the purpose intended.
24-Jan-00	Authorization to ship inactivated FIV vaccines to Japan was not granted based on the info.i.e. VS Code ?, serial numbers.
14-Jan-00	Submitted letter to Dr. Carr requesting permission to ship two experimental inactivated FIV vaccines to Japan.
14-Feb-00	Submitted complete revision of Production Outline in response to USDA comments.
13-Mar-00	Submitted response to 1/24/00 letter regarding permission to ship FIV to Japan. Need to clarify if there is a VS Code, serial number, etc.
21-Mar-00	Approved the request to ship FIV inactivated vaccines to Japan with comments.
17-Apr-00	Submitted new FIV post inactivation virus testing Special Outline 217.
19-Apr-00	SO 217 returned unprocessed.
3-Jan-01	Submitted letter requesting permission to ship experimental vaccines of FIV & FIV/FeLV to Japan.
9-Jan-01	Authorization received to ship experimental products of FIV & FIV/FeLV to Japan.
16-Jan-01	Submitted new FIV post inactivation testing SO responding to USDA 2015 comments dated 4/19/00.
15-May-01	Submitted preliminary field safety trial protocol.
17-May-01	Submitted Immuno report & included a disk with statistical analysis.
17-May-01	Approved subtype A MCS X+25 infected cell line for use in production.
17-May-01	Approved subtype A infected cell line for use in production.
17-May-01	Approved the FIV subtype D infected cell line MCS & X+25 for use in production.
19-Jun-01	SO was approved w/ pen-and-ink changes and comments.
19-Jun-01	Submitted in-vitro report for FIV.
19-Jun-01	Approved the preliminary field trial protocol w/comments. A form for owners with adverse events/daily observations column with clear instructions. Our final report should state number of properly completed forms returned to cooperating veterinarians.
19-Jun-01	Approved the complete revision of the Outline (in response to USDA comments 2/14/00) with comments and pen-and-ink changes.
21-Jun-01	Obtained field trial authorization from state vets.
25-Jun-01	Submitted revised outline of production.
26-Jun-01	Submitted new SO 237 for ELISA potency test for FIV.
5-Jul-01	No response required to comments re: owners completing forms during field trial.
5-Jul-01	Submitted letter responding to comment re: owners completing forms during field trial study.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
16-Jul-01	Submitted request for field safety test w/ 2008s for 2 of the 3 prelicensing serials. (Notify AR, VA, MD, CA of any adverse reactions—see state letters for their individual request.)
23-Jul-01	State vet approvals received.
24-Jul-01	Submitted letter advising of corrected address for Missouri site (Dr. Roger Sifferman) and copies of state approvals.
25-Jul-01	7/16/01 2008s for Serials 129050A & 129051A are filed as satisfactory. Verbal authorization on 7/20/01 to initiate field safety trial and ship 2000 doses of each serial. Amended protocol is approved.
25-Jul-01	USDA responded to the 7/5/01 ltr re: owners completing forms & their response was daily observation records from cat owners will not be required. Field safety protocol is adequate.
25-Jul-01	The change in address for the Missouri site was noted by USDA.
22-Aug-01	Immuno report filed as satisfactory.
30-Aug-01	Submitted ltr to Dr. Ludemann re: additional investigator for state of CA.
4-Sep-01	Verbal approval to ship to the additional investigator for the state of CA.
20-Sep-01	Submitted 2008 for 3rd PLS and requested TA#.
24-Sep-01	Submitted documentation of the storage temp of the Reference vaccine to support a 5 yr dating for frozen References. Approval of serial 1475-07-090299 (full dose) is contingent upon documentation (ltr of 8/22/01).
2-Oct-01	Received verbal TA#8279 to submit samples of the 3 PLS.
2-Oct-01	USDA approved new SO 237 ELISA potency.
3-Oct-01	USDA approved the revised Production Outline w/ comments & pen-and-ink changes.
5-Oct-01	Submitted samples of the 3 PLS to CVB-L under TA#8279 for confirmatory testing.
15-Oct-01	Reference 1475-07-090299 is approved for use as a Reference vaccine for serial release testing. The in vitro report satisfies concerns re: storage conditions of the immuno/ref serial. Need to submit FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 and approval of this report is contingent upon review of the subtype A infected cell line reaction w/ 1D9.
31-Oct-01	Submitted data for FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9.
2-Nov-01	The data submitted supporting the FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 was approved.
14-Nov-01	Submitted new label(s) (Spanish/English).
19-Nov-01	Submitted field trial report.
30-Nov-01	Changed FIV placebo vaccine lot# to 1516-62-031501. This lot was used in potency test validation and for all vaccine testing.
30-Nov-01	Submitted inactivation kinetics demonstrating that one inactivation procedure is sufficient for this virus per USDA 2015 comments dated 10/3/01.
4-Dec-01	Submitted "Supplemental Data - Addendum to the Demonstration of the Safety of FDAH's FIV Vaccine, KV in Cats Under Field Conditions". This should complete our submission for the field trial.
13-Dec-01	Submitted complete revision of SO 217.
18-Dec-01	Submitted results of study of experimental vaccines sent to Japan to CVB-L.
27-Dec-01	USDA approved the FIV placebo vaccine lot change sub'td on 11/30/01.
7-Jan-02	USDA approved the field trial report.
8-Jan-02	USDA approved the study of experimental vaccines sent to Japan.
9-Jan-02	USDA approved the inactivation kinetics.
9-Jan-02	CVB-L has completed satisfactory confirmatory testing of Prelicensing Serials 129050A, 129051A & 129052A.
4-Feb-02	Submitted new labels (FDAH requests the 10/22/01 & 11/14/01 submission be returned as sketches).
6-Feb-02	USDA approved labels submitted on 11/14/01 as sketches (per FDAH's request on 2/4/02).
21-Feb-02	Submitted new labels that replace sketches (FDAH requests the 2/4/02 submission be returned as sketches).
22-Feb-02	USDA approved labels submitted on 02/04/02 as sketches (per FDAH's request on 2/21/02).
25-Feb-02	Requested to ship up to 12 doses each of Serials 1749-20-122801 and 1749-25-122801 to Saitama, Japan.
13-Mar-02	USDA gave permission (verbal perm. given on 3/7/02) to ship up to 12 doses each of Serial Nos. 1749-20-122801 and 1749-25-122801 to Dr. Setsuo Arai of the Kitasato Institute, Saitama, Japan.
14-Mar-02	The Biological Product License was issued (we rec'd on 3/15/02).
14-Mar-02	USDA approved labels submitted on 2/21/02.
20-Mar-02	Submitted FDAH's press release to USDA.
21-Mar-02	Faxed a revised press release per telephone conversation between Dr. Ludemann & Dr. Steve Chu on 3/20/02.
22-Mar-02	USDA approved FDAH's press release sub'td on 3/20/02. This also confirms USDA's verbal approval on 3/21/02 (faxed) to add the patent acknowledgements for the University of California and the University of Florida.
22-Mar-02	Submitted efficacy report in 8-week-old kittens. (Cover ltr dated 4/1/02)
5-Apr-02	Submitted for USDA approval a press release to be distributed internationally.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
18-Apr-02	USDA approved the press release to be distributed internationally.
24-Apr-02	Submitted a quick synopsis on the FIV 6-month DOI. No response from USDA is required.
26-Apr-02	Submitted 2 separate letters ("Dear Distributor:" and "Dear Animal Health Industry Professional:") with advertising material attachments for USDA review and approval.
1-May-02	USDA gave verbal authorization (also confirmation letter dated 5/1/02) to distribute the advertising material identified as 2 letters.



ATTACHMENT B

United States  
Department of  
Agriculture

Marketing and  
Regulatory  
Programs

Animal and Plant  
Health Inspection  
Service

Veterinary Services

Center for Veterinary  
Biologics  
Suite 104  
510 South 17th Street  
Ames, IA 50010  
(515) 232-5785  
FAX (515) 232-7120

Federal Relay Service  
(Voice/TTY/ASCII/  
Spanish)  
1-800-877-8339



OVERNIGHT MAIL  
March 14, 2002

Ms. Madonna Carlson  
Fort Dodge Laboratories  
800 5th Street, NW  
P.O. Box 518  
Fort Dodge, IA 50501

Dear Ms. Carlson:

Enclosed is a new United States Veterinary Biological Product License issued this date to American Home Products Corporation, Establishment No. 112, authorizing production of the following:

Feline Immunodeficiency Virus Vaccine, Killed Virus, Code 15A5.21

Please note the restrictions under which this license is issued.

This U.S. Veterinary Biological Product License does not constitute a patent license. If this product or technology used in the manufacture of this product has been patented or is pending patent, the licensee should obtain a patent license from the patent owner.

If this license does not agree with your records, please return it to this office with your comments.

Sincerely,

Richard E. Hill, Jr., D.V.M.  
Director  
Center for Veterinary Biologics

Enclosure

cc: Bio Regulatory Affairs  
FILE: VS Code 15A5.21  
Product License Book

(see Bio Reg Notice #13 for  
distribution list)

**RECEIVED**

MAR 15 2002  
BIOLOGICAL  
REGULATORY AFFAIRS



# United States Department of Agriculture

## UNITED STATES VETERINARY BIOLOGICAL PRODUCT LICENSE

Washington, D.C.

This is to certify that, pursuant to the terms of the Act of Congress approved March 4, 1913 (37 Stat. 832), governing the preparation, sale, barter, exchange, shipment, and importation of viruses, serums, toxins, and analogous products intended for use in the treatment of domestic animals, the person holding United States Veterinary Biologics Establishment License No. 112 is authorized to prepare in the facilities designated in the establishment license.

### FELINE IMMUNODEFICIENCY VIRUS VACCINE

Killed Virus

Code 15A5.21

Preparation shall be in accordance with the provisions of the Act, the regulations made thereunder, and additional restrictions or requirements when listed below.

1. For use by or under the supervision of a veterinarian.
2. Marketing and promotional materials must be submitted to the Center for Veterinary Biologics for review and approval prior to use.

This license is subject to termination as provided in the regulations made under the authority contained in said Act, and to suspension or revocation if the licensee violates or fails to comply with said Act or the regulations made thereunder.

March 14, 2002

Date



Director, Center for Veterinary Biologics  
Animal and Plant Health Inspection Service



US005275813A

**United States Patent [19]**

Yamamoto et al.

[11] Patent Number: **5,275,813**[45] Date of Patent: **Jan. 4, 1994**

[54] **METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS**

[75] Inventors: Janet K. Yamamoto, Hercules; Niels C. Pedersen, Winters, both of Calif.

[73] Assignee: The Regents of the University of California, Oakland, Calif.

[21] Appl. No.: 739,014

[22] Filed: Jul. 31, 1991

Yamamoto et al. (1988) Leukemia, Dec. Supp. 2:204S-215S.

Yamamoto et al. (1988) Am J. Vet. Res. 49:1246-1258.

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Desrosiers et al. (1989) PNAS USA 86:6353-6357.

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Berman et al. (1990) Nature 345:622-625.

*Primary Examiner—Christine M. Nucker*

*Assistant Examiner—D. Barnd*

*Attorney, Agent, or Firm—Townsend and Townsend*

*Khourie and Crew*

[57] **ABSTRACT**

Compositions derived from a novel viral isolate designated feline immunodeficiency virus (FIV) include the whole virus, proteins, polypeptides and, polynucleotide sequences derived from the virus; and antibodies to antigenic sites on the virus. These compositions are useful in a variety of techniques for the detection of and vaccination against FIV. Detection methods disclosed include immunoassays for both the virus and antibodies to the virus, and the use of polynucleotide probes to detect the viral genome. Vaccines include both wholly and partially inactivated viruses inactivated cell lines expressing FIV antigens, and subunit vaccines. Whole, live virus is also useful as a model system for predicting the behavior of human immunodeficiency virus (HIV).

3 Claims, 11 Drawing Sheets

**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 618,030, Nov. 16, 1990, Pat. No. 5,037,753, which is a continuation of Ser. No. 89,700, Aug. 26, 1987, abandoned.

[51] Int. Cl.<sup>5</sup> ..... A61K 39/12

[52] U.S. Cl. ..... 424/89

[58] Field of Search ..... 424/89; 435/240.2, 235.1

[56] **References Cited****FOREIGN PATENT DOCUMENTS**

WO90/13573 11/1990 PCT Int'l Appl. .... C07K 13/00

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Berzofsky, J. A. et al. (1991), J. AIDS 4:451-459.

Pedersen et al. (1987) Science 235:790-793.

American Assoc. for Can. Res., May 23, 1987, Ab. No. 3337.

The 3rd Int'l. Conf. on AIDS, Jun. 1-5, 1987.

Yamamoto et al. Fed. Amer. Soc. for Experimental Biology, Apr. 2, 1987.

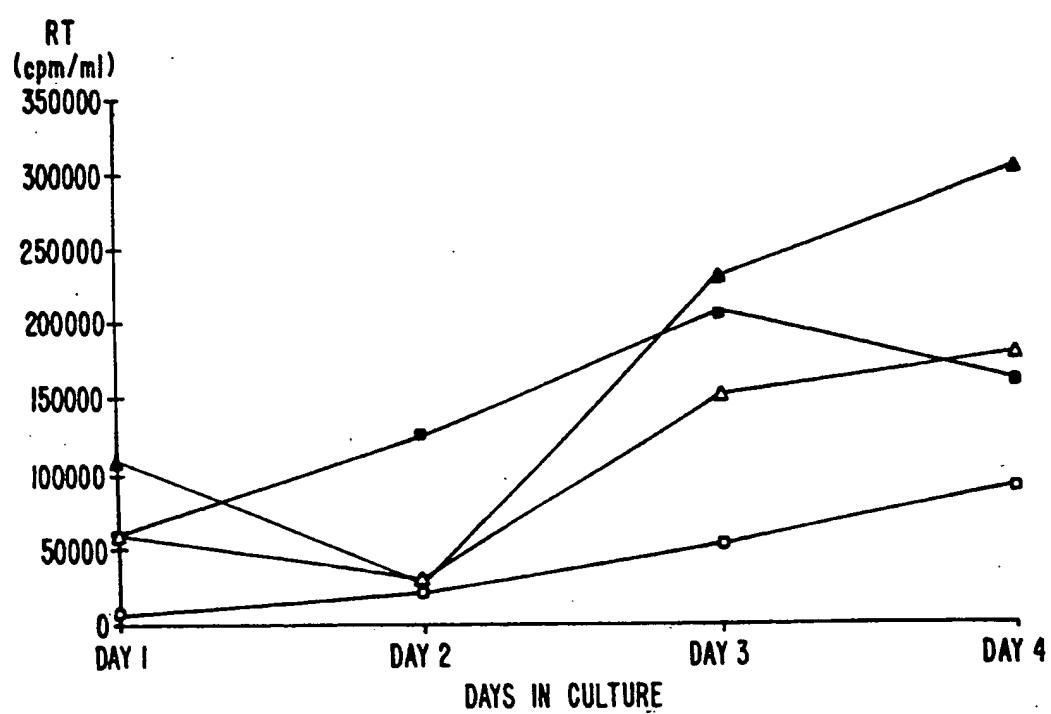
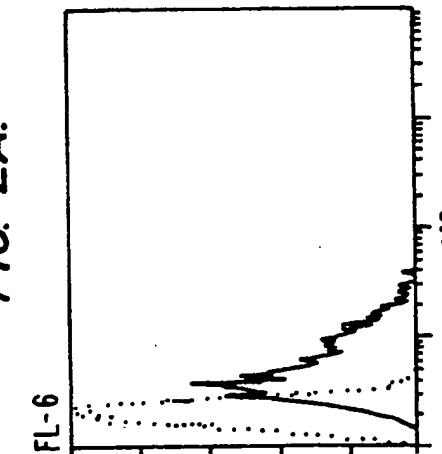
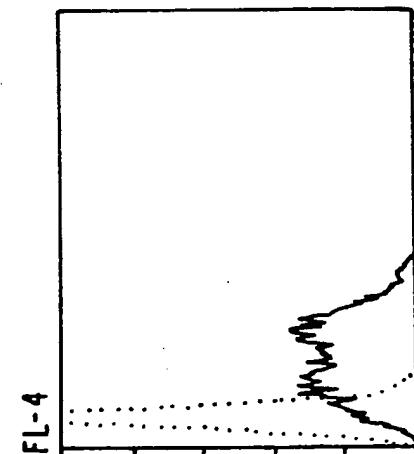
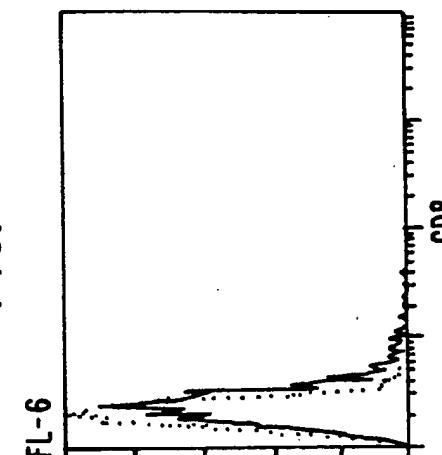
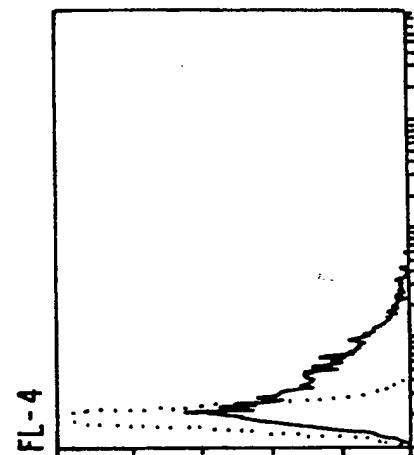
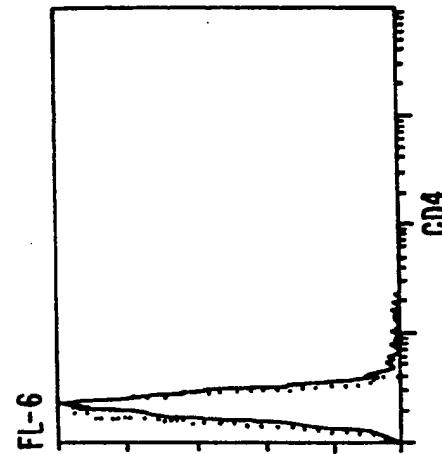
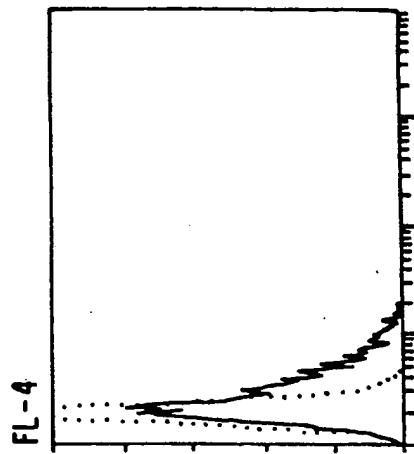
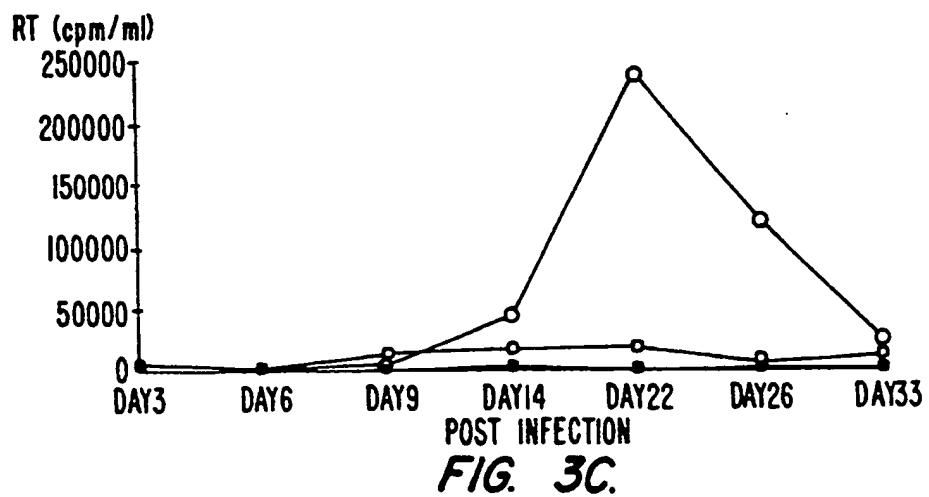
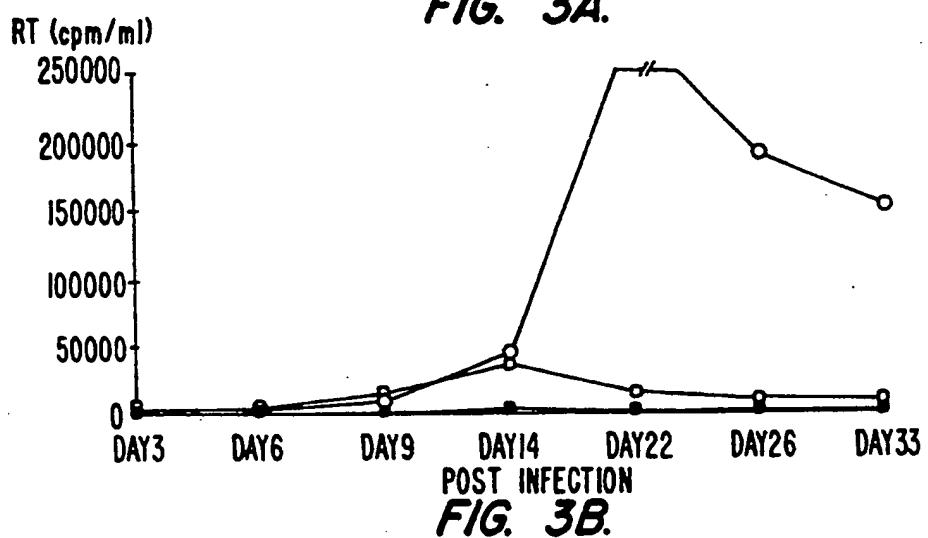
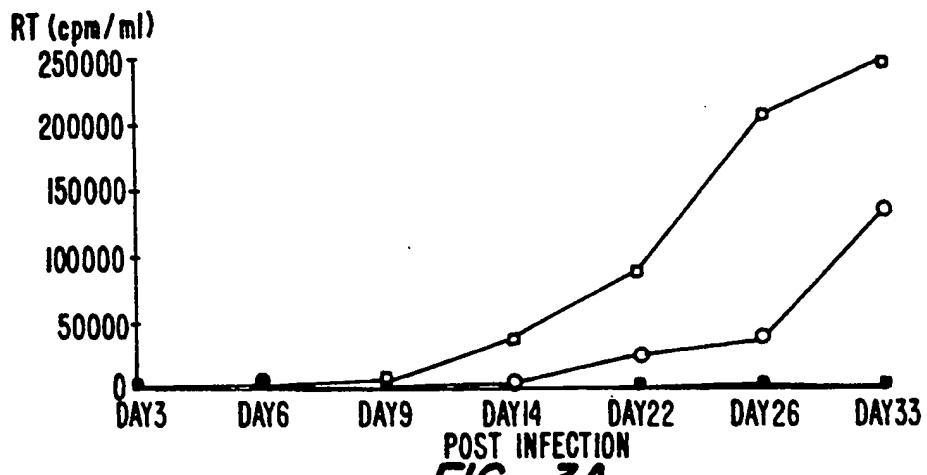
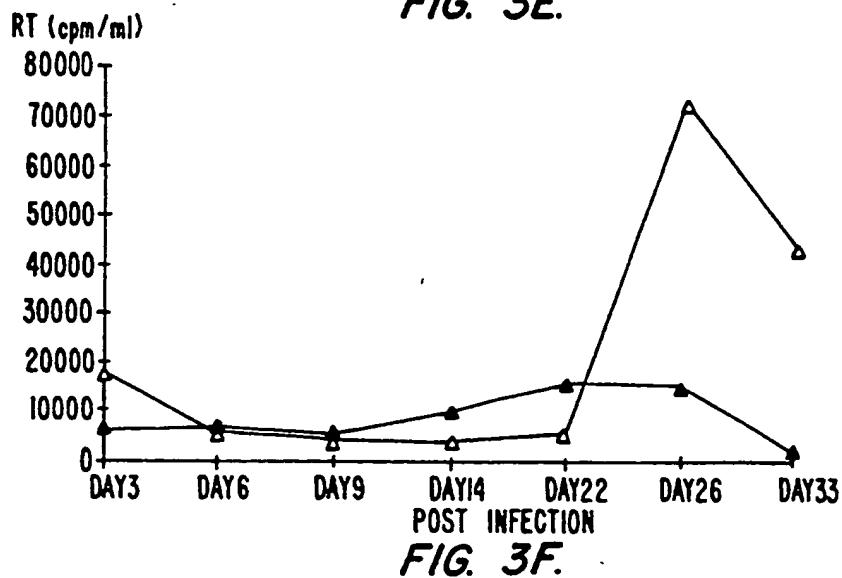
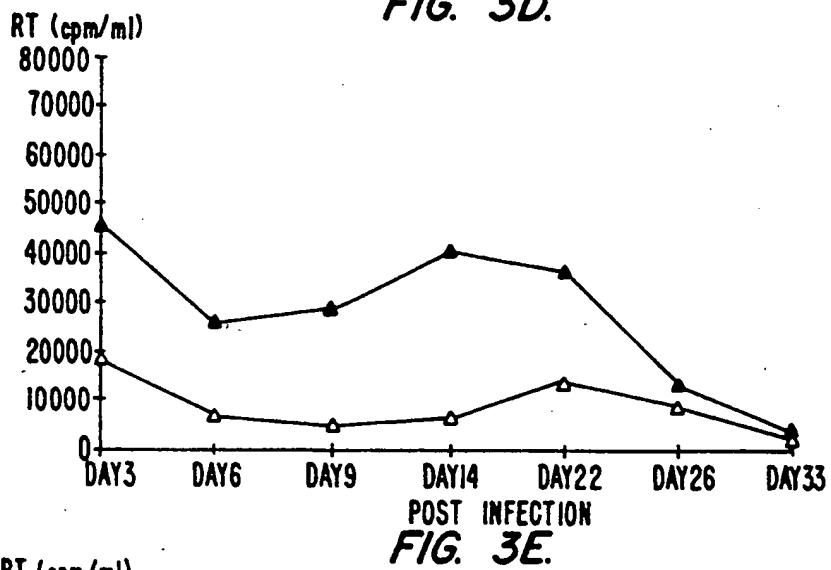
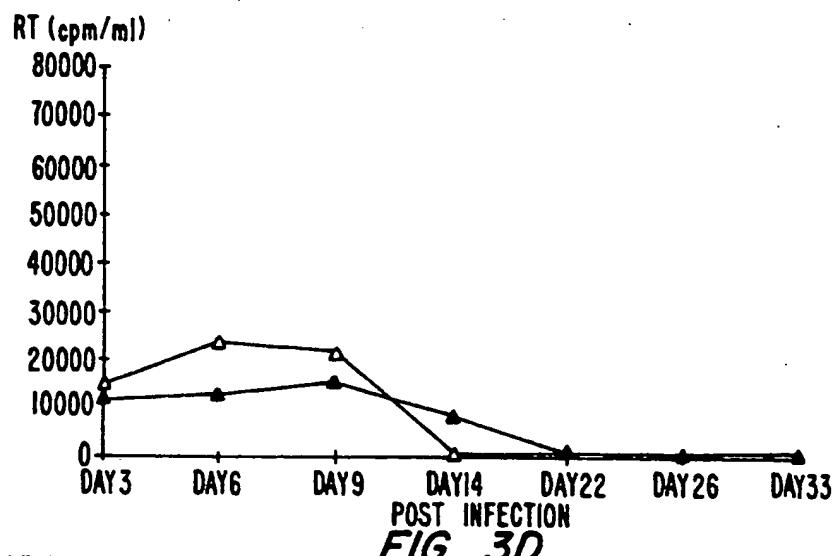


FIG. 1.







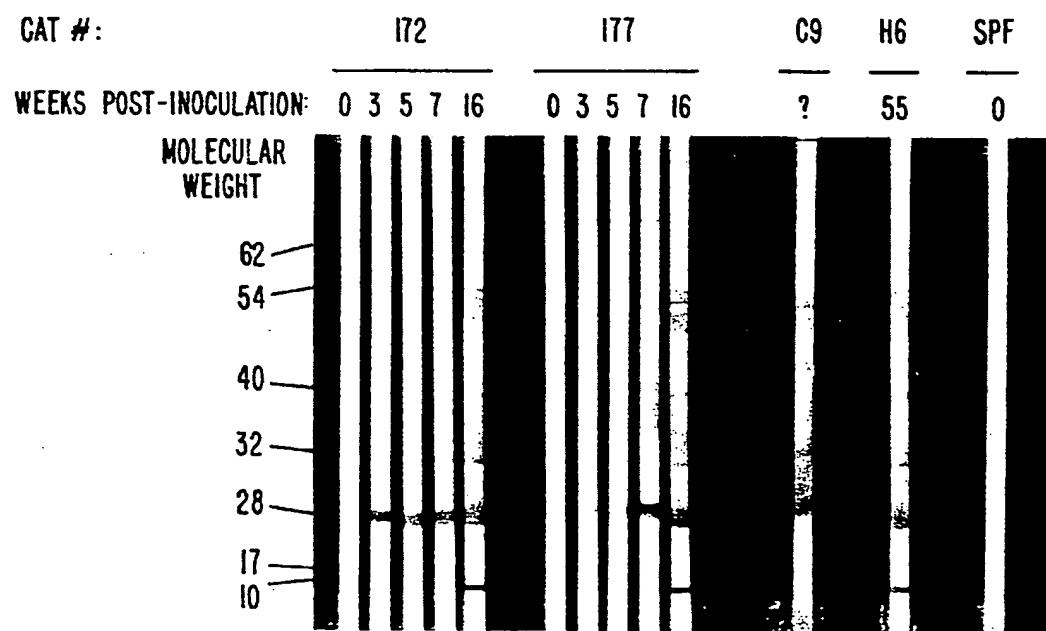


FIG. 4.

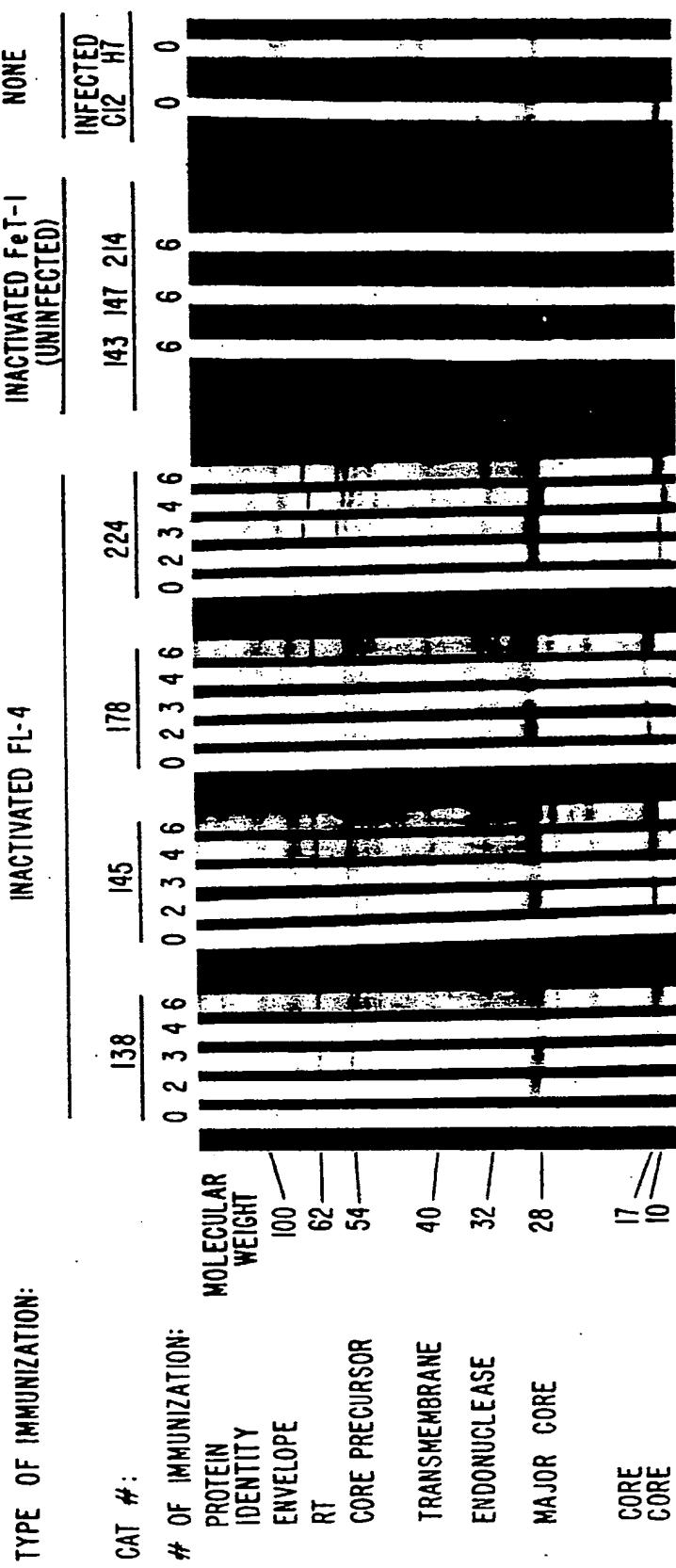


FIG. 5A.

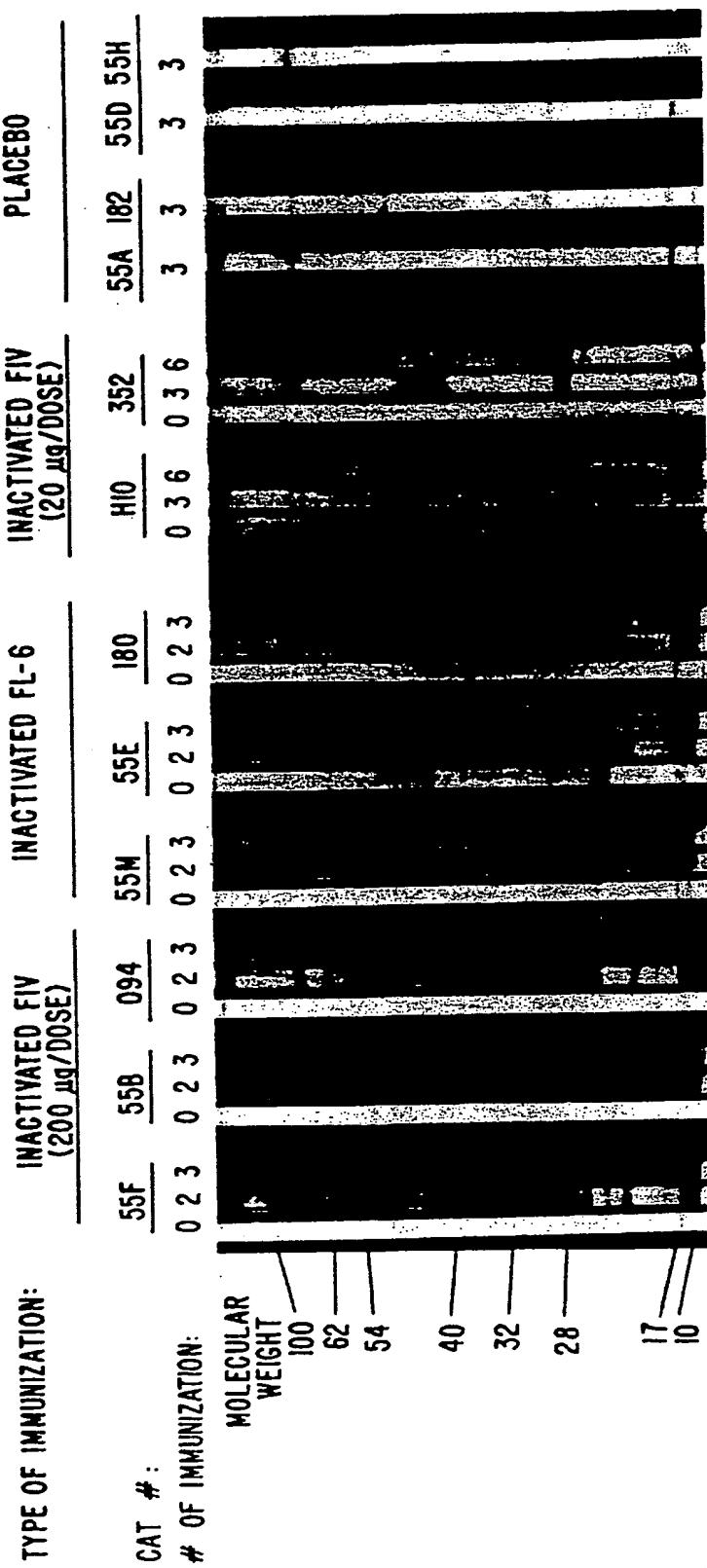


FIG. 5B.

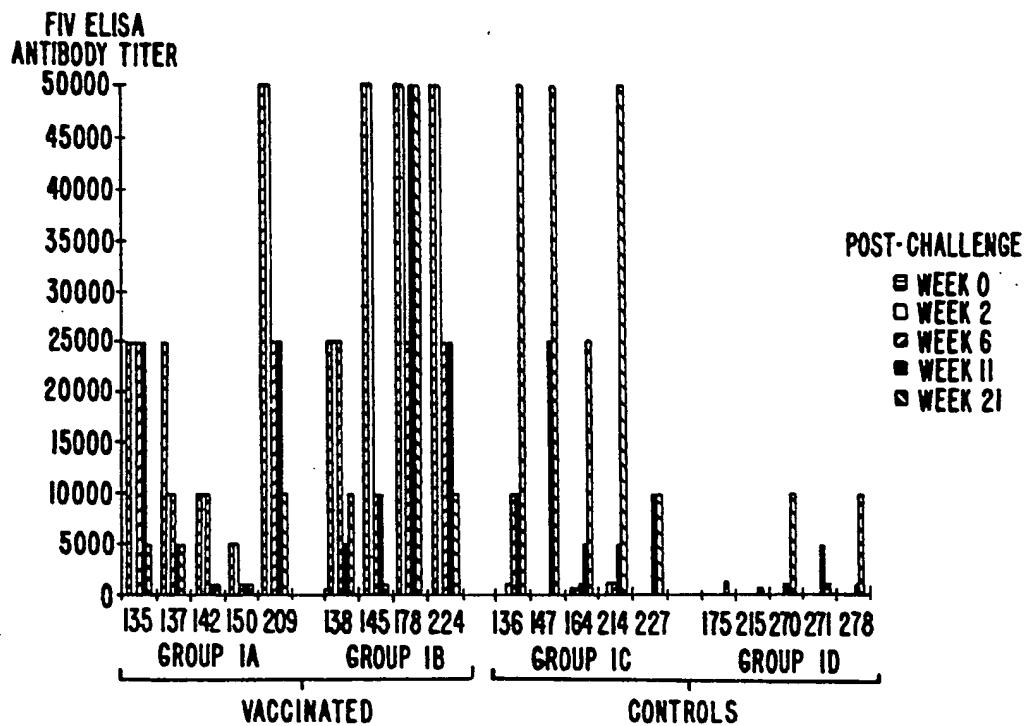


FIG. 6A.

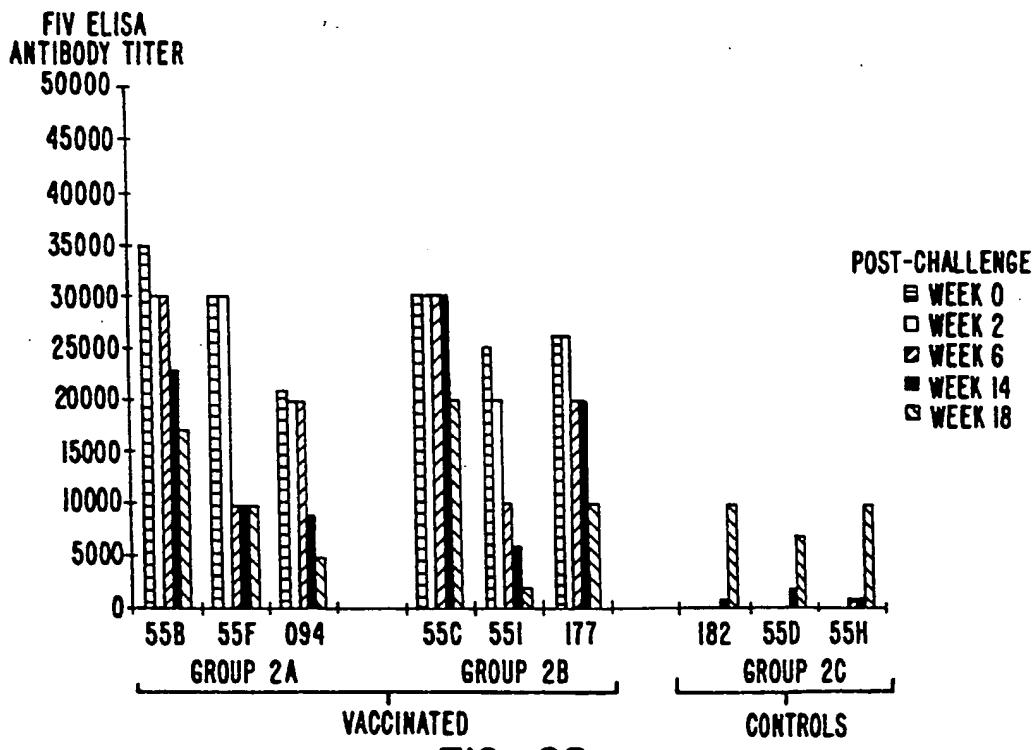


FIG. 6B.

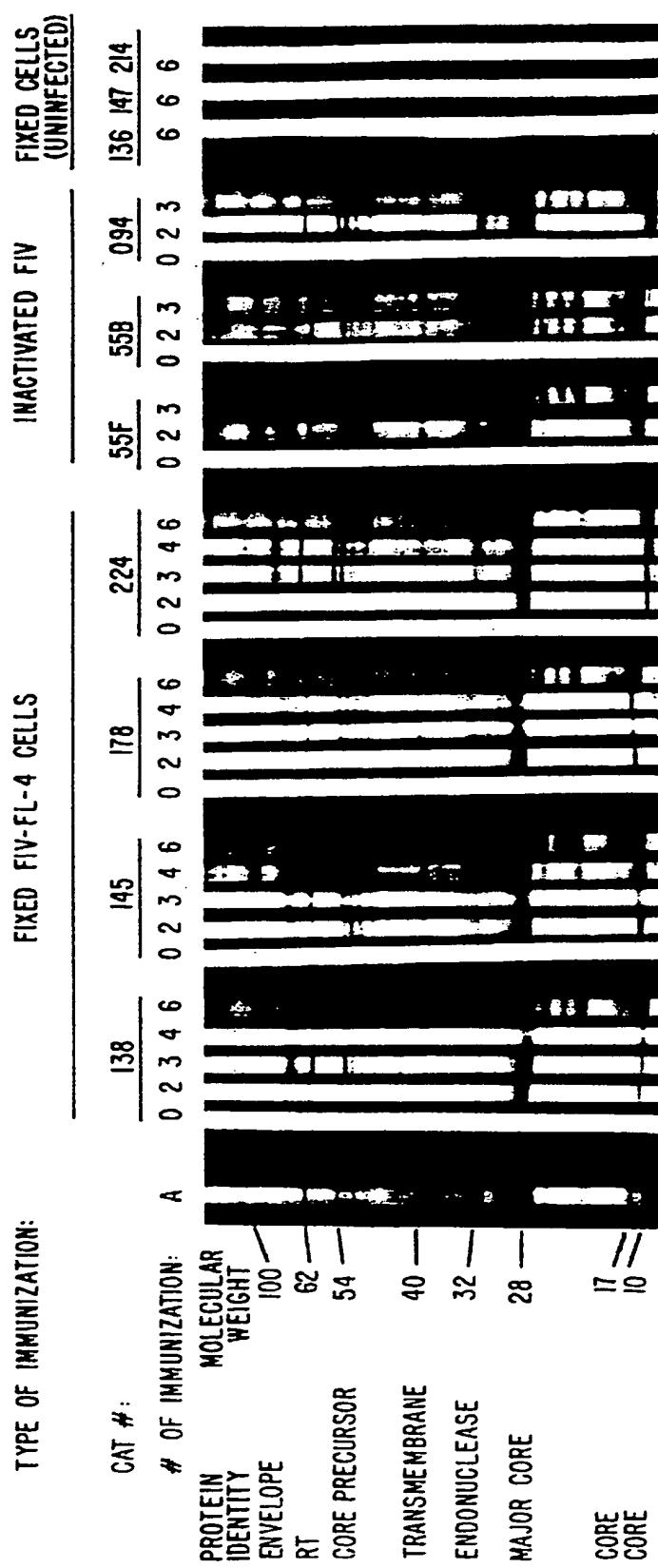


FIG. 7.

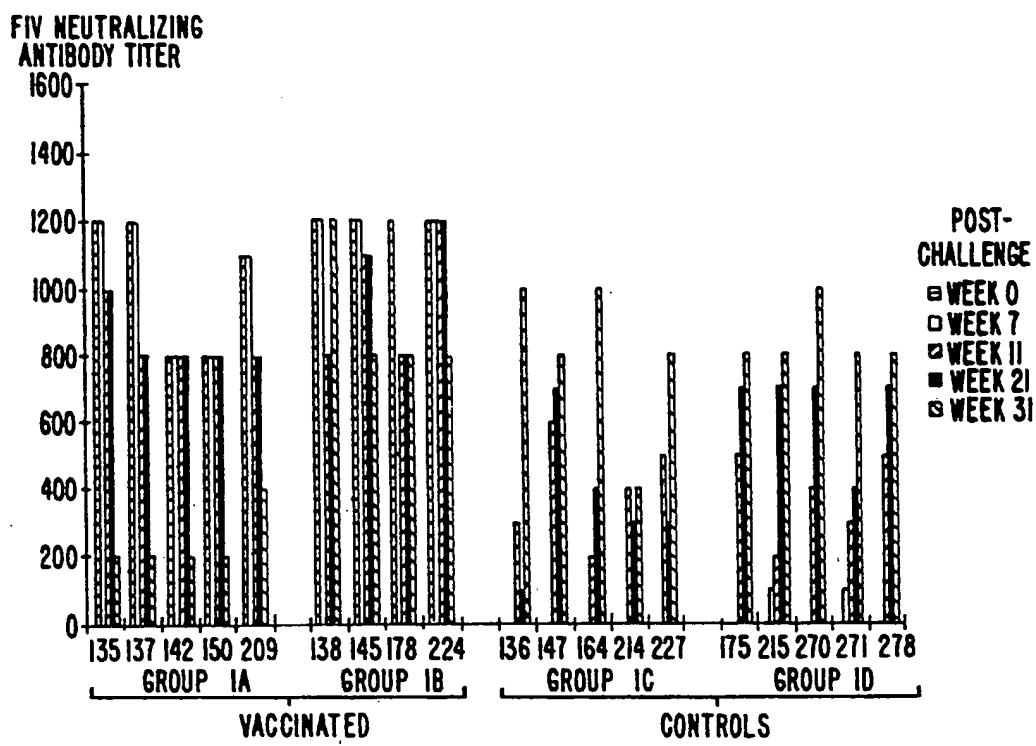


FIG. 8A.

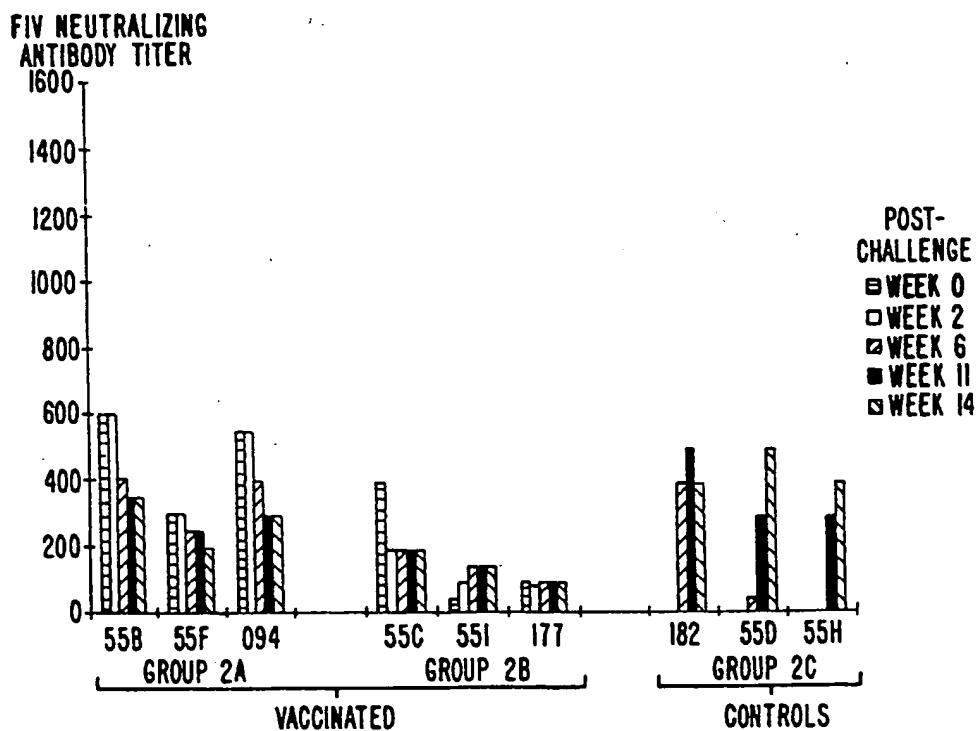


FIG. 8B.

## PROLIFERATION RESPONSE

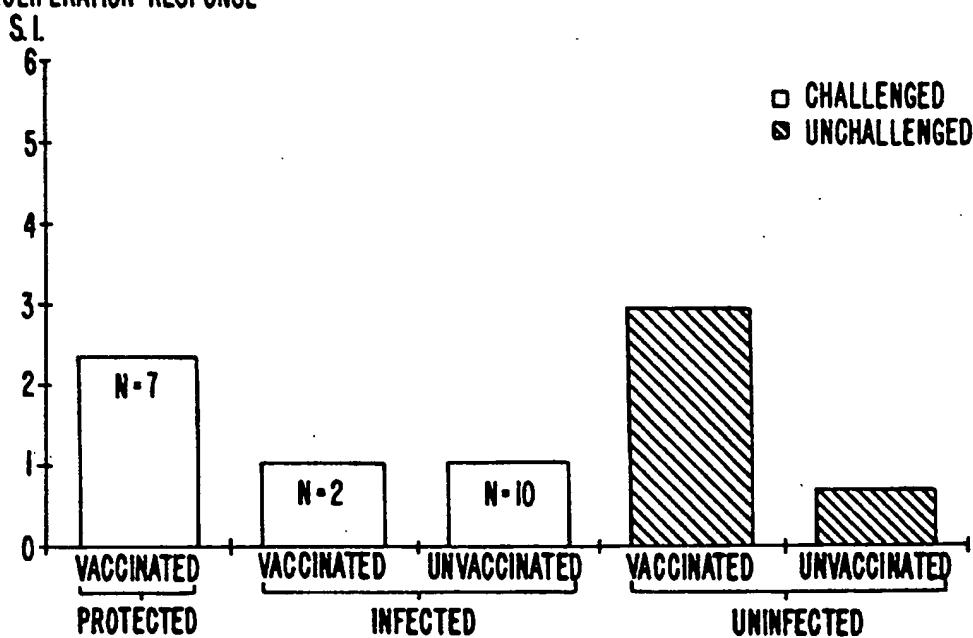


FIG. 9A.

## IL 2 INDUCTION

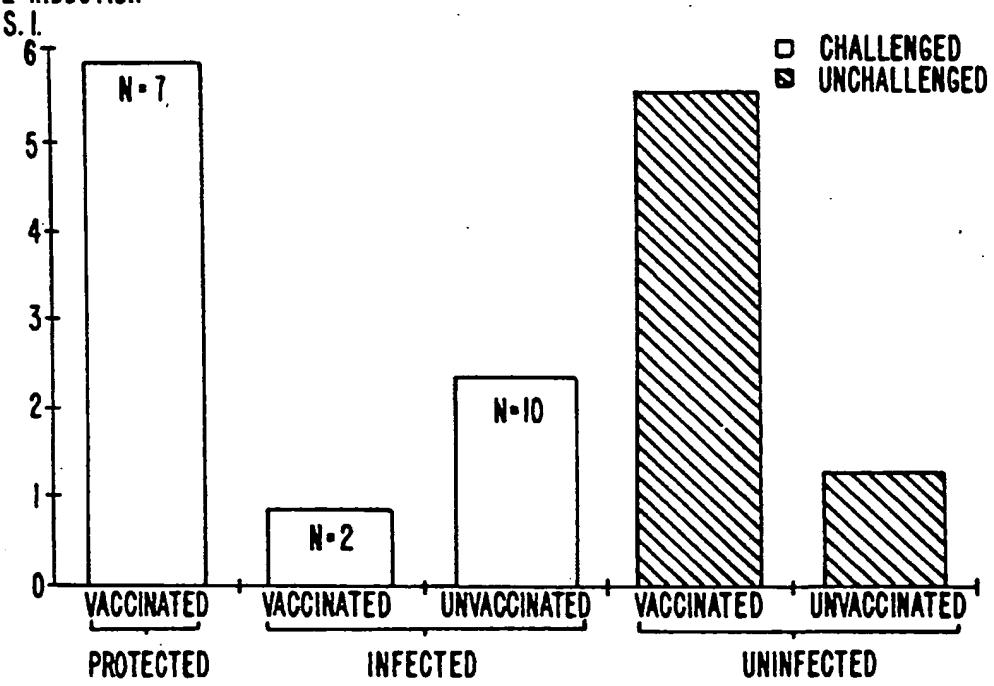


FIG. 9B.

## METHODS AND COMPOSITIONS FOR VACCINATING AGAINST FELINE IMMUNODEFICIENCY VIRUS

This invention was made with Government support under Grant No. CA 39016 awarded by the National Institute of Health. The Government has certain rights in this invention.

The present invention is a continuation-in-part of application Ser. No. 07/618,030, filed on Nov. 16, 1990, now U.S. Pat. No. 5,037,753, which was a continuation of application Ser. No. 07/089,700, filed on Aug. 6, 1987, now abandoned. The disclosures of both these applications are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates generally to the detection and treatment of viral infection. More particularly, the invention relates to compositions and methods useful for the diagnosis of and vaccination against infection with a newly-discovered lymphotropic retrovirus, initially designated as feline T-lymphotropic lentivirus and presently designated feline immunodeficiency virus (FIV).

Domestic cats may become infected with several retroviruses, including feline leukemia virus (FeLV), feline sarcoma virus (FeSV), endogenous type C oncovirus (RD-114), and feline syncytia-forming virus (FeSFV). Of these, FeLV is the most significant pathogen, causing diverse symptoms, including lymphoreticular and myeloid neoplasms, anemias, immunemediated disorders, and an immunodeficiency syndrome which is similar to human acquired immune deficiency syndrome (AIDS). Recently, a particular replication-defective FeLV mutant, designated FeLV-AIDS, has been more particularly associated with immunosuppressive properties.

While immunodeficiency syndrome in cats has normally been associated with FeLV, immunodeficiency-like symptoms have been observed in cats which are seronegative for FeLV, usually without alternative explanation. It would be desirable to identify etiological agents other than FeLV which are responsible for causing immunodeficiency in cats. It would be particularly desirable to provide methods and compositions for the detection of and vaccination against such newly-identified etiological agents, and in particular, against FIV.

#### 2. Description of the Background Art

The discovery of feline T-lymphotropic lentivirus (now designated feline immunodeficiency virus) was first reported in Pedersen et al. (1987) Science 235:790-793 Abstracts concerning the discovery of the virus have been presented at the American Association for Cancer Research on May 23, 1987 (Abstract No. 3337); and The Third International Conference on Acquired Immune Deficiency Syndrome, Jun. 1-5, 1987. A poster concerning discovery of the virus was presented at a meeting of the Federation of American Society for Experimental Biology on April 2, 1987.

Characteristics of FIV have been reported in Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S; Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258; and Ackley et al. (1990) J. Virol. 64:5652-5655. Cloning and sequence analysis of FIV have been reported in Olmsted et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:8088-8092 and 86:4355-4360; and

Talbott et al. (1989) Proc. Natl. Acad. Sci. U.S.A. 86:5743-5747. Hosie and Jarret (1990) AIDS 4:215-220, describes the serological response of cats infected with FIV.

5 A portion of the experimental data presented in this application was published in AIDS 1990 4 (Suppl. 1):S163-S165.

Inactivated cell-virus and cell-free whole simian immunodeficiency vaccines have been reported to afford protection in macaques (Stott et al. (1990) Lancet 336:1538-1541; Desrosiers et al. PNAS U.S.A. (1989) 86:6353-6357; Murphrey-Corb et al. (1989) Science 246:1293-1297; and Carlson et al. (1990) AIDS Res. Human Retroviruses 6:1239-1246). A recombinant HIV gp120 vaccine has been reported to afford protection in chimpanzees (Berman et al. (1990) Nature 345:622-625).

### SUMMARY OF THE INVENTION

Compositions and methods are provided for vaccination against a novel feline retrovirus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV). The compositions include vaccines comprising an immunogen capable of eliciting an immune response protective against infection by FIV when administered in an effective amount to a susceptible host. The immunogen will display determinant sites characteristic of the virus, such as those found on the major envelope and core proteins. The preferred immunogens include inactivated whole virus, attenuated whole virus, and inactivated cell lines infected with FIV and which express FIV antigens on their surface. Other immunogens which may find use include polypeptides which mimic the determinant sites, such as FIV peptides (to produce subunit vaccines), antiidiotype antibodies, and the like.

According to the method of the present invention, the vaccine compositions are administered to susceptible hosts, usually cats, in amounts effective to afford immunity against subsequent challenge by FIV. The vaccines may be administered by any conventional route, including subcutaneously, intramuscularly, and oranasally, and will usually be administered at least twice over intervals spaced-apart by one or more weeks to achieve the desired immunity.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: FL-4 ( $\blacktriangle$ ), FL-6 ( $\Delta$ ), FIV-FeT1 ( $\blacksquare$ ), and FIV-CRFK ( $\square$ ) cells were seeded at  $5 \times 10^5$  cells/ml tested daily for the RT activity in their culture fluids. A gradual increase in RT activity was observed over the four days of culture, with peak RT titers detected on Day 4 for all cell cultures except FIV-FeT1 which had it on Day 3. Peak viable cell counts ( $1.0-2.25 \times 10^6$  cells/ml) were observed on Day 3 for all cell cultures except for the FIV-FeT1 culture which had its peak viable cell count ( $1.3 \times 10^6$  cells/ml) on Day 2. The percent cell viabilities during the four days of culturing were 75-90% for FIV-FL-4, 70-90% for FIV-FL-6, 70-80% for FIV-CRFK, and 55-65% for FIV-FeT1.

FIGS. 2A-2F: The FACS profiles of the surface phenotype of FL-4 (FIGS. 2A, 2B, and 2C) and FL-6 (FIGS. 2D, 2E, and 2F) were determined using characterized monoclonal antibodies to feline CD4 (Fel 7), CD8 (FT2), pan T-cell (F42) and the feline light chain and  $\mu$  heavy chain specific (AC5) markers. Both cell lines had cell populations which were positive for CD4 (FIGS. 2C and 2F), CD8 (FIGS. 2B and 2E), and pan T-cell (FIGS. 2A and 2D). Both FL-4 and FL6 cells

tested negative by FACS analysis for surface B cell markers using monoclonal antibodies (AC5) (data not shown). The solid lines represent the FACS profiles of FL-4 and FL-6 cells and the dotted lines represent the FACS profiles of negative control cells. The percentages of FL-4 cells that were positive for CD4, CD8 and pan T-cell markers were 10%, 20%, and 80% respectively. The percentages of FL-6 cells that were positive for CD4, CD8 and pan T-cell markers were <8%, 11%, and 76%, respectively. Depending on the culture conditions, expression of CD4 and CD8 on the cell membrane can be decreased or eliminated. The abscissa represents fluorescence intensity and the ordinate represents relative cell number.

FIGS. 3A-3F: FIV from FL-4 (A,D), FIV-FeT1 (B,E) and FIV-CRFK (C,F) cells were tested for their infectivity on different feline PBLs (A,B,C) and feline thymocytes (D,E,F). Uninfected feline lymphoid cells used in this study were FeT1.1 (■), FeT1.2 (□), FeT1.3 (○), Thy1 (Δ), and Thy2 (▲). All of the FeT1 cells were derived from uninfected PBLs and Thy cells were primary thymocytes obtained from FIV-free kittens. FeT1.1, FeT1.2, and FeT1.3 were subclones of the uninfected FeT1 line, which was the precursor line for FIV-FeT1 cells. The percentage of cells that was positive for CD4 and CD8 markers was <2% and 5% for FeT1.1, <2% and <2% for FeT1.2, and <2% and 4% for FeT1.3, 54% and 4% for Thy 1, and 38% and <2% for Thy2, respectively. Interestingly, FIV from all cell lines were able to either transiently and persistently infect all lymphoid cells except for those from FeT1.2 cultures, whose cells also totally lacked the expression of both CD4 and CD8 markers. Another observation was that FIV from FL-4 and FIV-FeT1 cells infected thymocytes more rapidly but produced a lower titer of virus than those produced by FeT1.1 or FeT1.3 cells. The major difference between the thymocytes and the FeT1.1 or FeT1.3 cells was the large number of CD4+ cells present in the thymocyte cultures. Thus, this observation suggests that the rapid FIV infection of the thymocytes was correlated to the increased number of CD4+ cells.

FIG. 4: Immunoblot analysis was performed on the sera from cats inoculated with 2 ml of cell-free TCF (150,000 cpm/ml RT activity) from either FL-4 (Cat #172) or FL-6 (Cat #177) cultures. The FIV antibody development in these cats was similar to the progression observed previously in SPF cats inoculated with plasma or blood from FIV-infected cats or with TCF from primary PBL cultures (Yamamoto et al., (1988) supra.). Their immunoblot profiles at 16 weeks post-inoculation (pi) resembled those of sera from naturally (Cat #C9) or experimentally (Cat #H6) infected cats. The PBLs from these cats at 10 weeks pi were positive for FIV by virus isolation (data not shown). Both immunoblot and virus isolation results demonstrate that these cats were infected with FIV.

FIGS. 5A and 5B: The immunogenicity of the FIV produced from our FIV-infected cell lines was evaluated in cats. The reactivities of the antibodies produced in cats immunized with either inactivated FL-4 (A) or FL-6 (B) cells or with inactivated FL-4-produced virus (B) were determined by immunoblot analysis. Cats were immunized six-times with inactivated FL-4 or uninfected FeT1 cells and their serum immunoblot profiles were compared to those of serum from cats naturally (Cat #C12) or experimentally (Cat #H7) infected with FIV (A). Cats were also immunized four-times with

inactivated FIV (produced by FL-4 cells) and with inactivated FL-6 cells (B) and evaluated similarly. In the last study, cats were immunized eight-times with 20 µg per dose of inactivated FIV and these results presented. All immunizations were done at two week intervals except for the final interval between the fifth and sixth immunizations in the first study. The adjuvants used were either MDP (A) or a combination of Freund's complete and incomplete adjuvants (B). The predicted molecular masses of the FIV proteins, derived from nucleotide sequence analysis (Olmstead et al. (1989) supra., and Talbott et al. (1989) PNAS U.S.A. 86:5743-5747), have been identified as: 24.5-25.1 kD for major core protein, 14.7-14.9 kD for N-terminal gag protein (minor core protein), 9.5-9.6 kD for nucleocapsid protein (minor core protein), 49.2-49.5 kD for gag precursor protein (core precursor), 61.5 kD for reverse transcriptase (RT), 30.7 kD for endonuclease, 100 kD for major envelope glycoprotein (outer membrane), 36 kD for transmembrane glycoprotein, and 140 kD for precursor envelope glycoprotein. The banding profiles derived from radioimmunoprecipitation analysis using [<sup>3</sup>H]glucosamine (Olmstead et al. (1989) PNAS U.S.A. 86:4355-4360 and Hosie et al. (1990) AIDS 4:215-220) or [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine (O'Connor et al. (1989) J. Clin. Micro. 27:474-479), have demonstrated a gp100-120 band for envelope, a gp36-41 diffuse band for transmembrane, and a gp130-140 band for envelope precursor. The molecular weights of the viral protein components, as described by the above analyses, correspond to the immunoblot patterns of 24-28 kD for major core, 15-17 kD for minor core, 10 kD for minor core, 54-55 kD for core precursor, 62 kD for RT, 32 kD for endonuclease, 37-44 kD (diffuse band) for transmembrane, and 100-120 kD for envelope (Yamamoto et al. (1988) supra.; Hosie et al. (1990) supra.; and O'Connor et al. (1989) J. Clin. Micro. 27:474-479). In this study, the development of antibodies to major core protein p28 was observed prior to the development of antibodies to the envelope glycoprotein gp100 in both immunization studies (A and B). Our immunoblot analysis of the sera from immunized cats closely resembled the immunoblot profiles of FIV-infected cats previously published by our laboratories and others (Yamamoto et al. (1988) supra.; Hosie et al. (1990) supra.; and O'Connor et al. (1989) J. Clin. Micro. 27:474-479). Comparison of high dose (200 g) indicates that large amounts of viral proteins are required to adequately and rapidly induce FIV antibodies (B). The immunoblot numbers represent the cat identification numbers.

FIGS. 6A and 6B: The FIV IgG antibody titer was measured by enzyme-linked immunosorbent assay (ELISA) using 250 ng/microwell of sucrose-gradient purified FIV as substrate and biotinylated goat anti-cat IgG (Vector Laboratories, BA-9000) as conjugating antibody (Pedersen et al (1987) Science 235:790-793). Sera from the different bleeding dates of each cat were serially diluted and assayed simultaneously in a single test. The results are based on two separate ELISA testings. Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIG. 7: Immunoblot analysis was performed on sera at a final dilution of 1:50 from cats immunized with fixed cell-virus or inactivated whole-virus vaccines. Results presented are those from cats immunized with fixed FIV-FL-4 cells (Group 1B), inactivated FIV

(Group 2A), or fixed uninfected FeT1 cells (Group 1C). Lane A is an immunoblot profile of a SPF cat experimentally infected with FIV.

FIGS. 8A and 8B: The neutralizing antibody titers to FIV were assayed the FIV-susceptible feline lymphoid cell line FeT1. In brief, diluted samples of heat-inactivated serum (56° C. for 30 min) were incubated with 100 tissue culture infective doses (TCID<sub>50</sub>) of FIV (Petaluma strain) for 45 min at 37° C. in a 25-cm flask. The FeT1 cells were added to this mixture at a final concentration of 2 × 10<sup>5</sup> cells/ml. After three days of culturing, the cells were washed once with Hank's balanced salt solution to remove residual virus from the culture and then resuspended in fresh culture media (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 10 mM HEPES buffer, 50 µg/ml gentamicin, 1 × 10<sup>-5</sup>M 2-mercaptoethanol, and 100 U/ml human recombinant IL-2). Virus infection was monitored by Mg<sup>++</sup>-dependent RT assays of the culture fluid. The serum was considered positive for neutralizing antibodies when RT activity was ≤ 50% of the infected control culture which had no serum exposure. Nonspecific antiviral activity (i.e., interferon activity) was not detected in the heat-inactivated serum samples using the antiviral assay with vesicular stomatitis virus (Yamamoto et al (1986) *Vet. Immunol. Immunopathol.* 11:1-19). Part A gives the results from cats immunized with the fixed cell-virus vaccine and part B gives results from cats immunized with the inactivated whole-virus vaccine.

FIGS. 9A and 9B: Cellular immunity of the cats immunized with the fixed cell-virus vaccine was monitored by assaying the PBLs for their ability to proliferate (FIG. 9A) or produce IL-2 (FIG. 9B) upon stimulation with inactivated whole FIV (1.25% paraformaldehyde inactivated). The PBLs were isolated from blood harvested at 27 weeks pc from all challenged animals and at 14 weeks post-immunization from unchallenged cats. The proliferation assay consisted of <sup>3</sup>H-thymidine incorporation by PBLs (1 × 10<sup>5</sup> cells/microwell) upon stimulation with inactivated FIV (4.5 µm/microwell) for five days at 37° C. Similarly, 1.5 ml-cultures of PBL (1 × 10<sup>6</sup> cells/ml) were incubated with FIV antigens (50 µm/ml) for two days and the culture fluid was assayed for IL-2 titer. The IL-2 assay consisted of measuring the amount of <sup>3</sup>H-thymidine incorporation of the IL-2-dependent murine HT-2C cells in presence or absence of IL-2 containing samples (Gillis et al. (1978) *J. Immunol.* 120:2027-2032). When compared to infected, unvaccinated control cats, the vaccine protected cats and the vaccinated but unchallenged cats responded significantly (stimulation index ≥ 2.0) to FIV antigenic stimulation in both proliferation ( $P < 0.001$ ) and IL-2 induction ( $P < 0.001$ ) assays. The  $P$  value was derived by using two-tailed  $t$ -test. Part A presents the results from the proliferation assay and part B the results from the IL-2 induction assay.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A novel virus designated feline immunodeficiency virus (FIV), previously designated feline T-lymphotropic lentivirus (FTLV) has been discovered and isolated in substantially pure form. The virus is infectious in cats, causing a wide variety of symptoms, including abortion, alopecia, anemia, chronic rhinitis, conjunctivitis, diarrhea, emaciation, enteritis, gingivitis, hematochezia, neurologic abnormalities, periodontitis, and seb-

orrheic dermatitis. The course of the disease is usually fatal.

The etiology, pathogenesis, and morphology of FIV closely resemble those of human immunodeficiency virus (HIV) and simian T-lymphotropic virus III (SAIDS), which cause acquired immunodeficiency syndrome in humans and primates, respectively. FIV does not appear to be antigenically related to HIV or to SAIDS, but rather appears to be a species-adapted lentivirus that has existed in cats for some time. Preliminary surveys conducted by the inventors herein indicate that FIV infection in cats may be widespread, possibly accounting for a significant proportion of the immunodeficiency symptoms found in cats who are free from FIV infection.

FIV is a feline immunodeficiency virus characterized as a retrovirus, more specifically as a lentivirus, which is tropic for T-lymphocytes of the host which it infects. The virus is also characterized by horizontal transmission, and may further be characterized by vertical transmission in at least some cases.

It is expected that FIV is polymorphic, and reference to FIV in the present application is intended to encompass the entire FIV family, including a variety of strains which share substantial amino acid sequence and nucleotide sequence homology and which are immunologically related. Substantial amino acid sequence homology means at least about 75% homology, usually at least about 80% homology, and frequently 90% homology and above in at least some of the viral genes and proteins. For example, the env, gag, or pol regions may display the requisite homology, while the genome as a whole does not. In such cases, so long as the viruses are immunologically related, the viruses will be considered to be FIV within the ambit of the present invention.

By immunologically related it is meant that the various strains will display substantial serologic cross-reactivity with the newly-discovered strain which has been deposited. Serologic cross-reactivity is defined as the ability of an antiserum or antibodies specific for the deposited FIV strain to react with other FIV strains as well as the deposited strain. Usually, immunologically related strains will cross-react with antibodies specific for more than one epitopic site, usually more than five epitopic sites, and frequently ten or more epitopic sites.

Conveniently, FIV strains may be identified by Western blot analysis where purified virus is disrupted with a suitable detergent, e.g., sodium dodecyl sulfate, and separated on a slab gel by electrophoresis. The separated polypeptide bands are transferred from the gel to nitrocellulose filter paper and visualized with labelled antibody. The molecular weights of the various resolved bands may then be determined by comparison to known molecular weight standards. Substantial similarity between the Western blot analysis of an unidentified virus and that of a known FIV virus indicates that the unknown virus is likely an FIV virus.

Other FIV isolates have been characterized, indicating that the nucleotide sequence of the envelope gene varies by no more than about 15% among isolates. Such isolates, from different regions, are described in Masashi et al. (1990) In: Proc. 6th Intnl. Conf. AIDS, June 20-24, San Francisco, Abstract Th.A. 284 (Japanese isolate); Phillips et al. (1990) *J. Virol.* 64:4605-4613 (San Diego, California); Olmsted et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:2448-2452 (Petaluma, California); Talbot et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86:5743-5747 (Petaluma, California); Rigby et al. (1991)

In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, Cameron House, Loch Lomond, Scotland, May 28-31, page 42 (Scotland); and Siebelink et al. (1991) In: Proc. Intnl. Feline Immunology and Immunodeficiency Workshop, supra. (The Netherlands). Any of these isolates could be used for preparing vaccines and cell lines according to the present invention.

FIV encodes an RNA-dependent DNA polymerase (reverse transcriptase) which is Mg.<sup>+2</sup>-dependent with maximal activity occurring at a Mg.<sup>+2</sup> concentration of approximately 5 mM and pH of approximately 7.8. FIV bands at a density of about 1.15 g/cm<sup>3</sup> in a continuous sucrose gradient. Western blotting of FIV-infected cell lysate yields major bands at approximately 22 to 28 kD, usually about 26 kD; 50 to 60 kD, usually about 55 kD; and 28 to 36 kD, usually about 32 kD.

FIV may be isolated from the sera of infected cats by conventional techniques. For example, peripheral blood lymphocytes (PBL) may be isolated from the blood of infected cats and placed in suitable culture media. The cultures are incubated, with normal PBL's being periodically introduced to the culture in order to maintain its viability as the original cells are killed by the virus. The infected cells should be placed in fresh culture medium periodically, and the virus may be recovered from the supernatant of the cell culture by sucrose-gradient separation, or other known separation techniques.

The FIV may also be obtained from other specimens, particularly from the lymph tissues of infected animals. The lymph tissues are broken and then suspended in culture medium, and the procedures described above are then carried out.

Compositions according to the present invention include the whole virus, as well as portions of the virus. The whole virus may be maintained in in vitro culture, as described above, or may be viably frozen at a temperature at or below about -78° C. (solid CO<sub>2</sub>-dry ice), usually in the presence of agents which promote amorphous, vitreous solidification rather than crystallization. Suitable agents include glycerol and dimethylsulfoxide. Portions of the FIV of particular interest include the structural and regulatory proteins encoded by the FIV genome, including the envelope and core proteins, and fragments thereof.

The FIV may also be maintained in chronically infected cell lines, particularly T-cell lines, as described in detail in the Experimental section hereinafter. For example, interleukin 2 (IL-2)-dependent T-cell lines can be infected with FIV and maintained in IL-2-supplemented culture media. IL-2-independent cell lines can then be prepared by repeated subculturing with a gradual depletion of IL-2. Surviving cultures can then be maintained in culture free from IL-2. The IL-2-independent FIV-infected cell lines have been found to possess enhanced viability and a reduced percentage of syncytial cells when compared to IL-2-dependent FIV-infected cell lines. See, Experimental section hereinafter.

The FIV used for infecting the cell lines may be isolated from infected cats, as described above, or may be obtained from the deposited Petaluma strain of the virus (A.T.C.C. VR 2186).

Particular non-infected feline T-lymphocyte cell lines (IL-2 dependent) which may be infected to produce chronically FIV-infected cell lines are designated FeT-1M (A.T.C.C. Accession No. CRL 10775) and FeT-2D

(A.T.C.C. Accession No. CRL 10774), both deposited at the American Type Culture Collection, Rockville, Maryland, on Jun. 7, 1991.

Particular FIV-infected cell lines (IL-2 independent) which have been established from FeT-1M are FL-4 (A.T.C.C. Accession No. CRL 10772) and FL-6 (A.T.C.C. Accession No. CRL 10773), both deposited at the American Type Culture Collection on Jun. 7, 1991. Both these cell lines have been found to be prolific producers of FIV.

FeT-1M, FeT-2D, FL-4 and FL-6 were developed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

Polypeptides of the present invention will be either haptenic or antigenic, including at least six amino acids, usually at least nine amino acids, and more usually twelve or more amino acids found contiguously within one of the natural FIV proteins. Polypeptides will generally correspond to at least one epitopic site which is characteristic of FIV. By characteristic, it is meant that the epitopic site will allow immunologic detection of the virus in a physiological sample with reasonable assurance. Usually, it will be desirable that the epitopic site be immunologically distinct from (i.e., not cross-reactive with antibodies which recognize) viruses other than FIV. In some cases, however, it may be desirable that the epitopic site be immunologically similar to other viruses.

The FIV polypeptides may be natural, i.e., including the entire FIV protein or fragments thereof isolated from a natural source, or may be synthetic. The natural polypeptides may be isolated from the whole virus which is obtained as described above by conventional techniques, such as affinity chromatography. Conveniently, polyclonal or monoclonal antibodies obtained according to the present invention (as described in more detail hereinbelow) may be used to prepare a suitable affinity column by well-known techniques. Such techniques are taught, for example, in Hudson and Hay, *Practical Immunology*, Blackwell Scientific Publications, Oxford, United Kingdom, 1980, Chapter 8.

Synthetic polypeptides which are immunologically cross-reactive with a natural FIV protein may be produced by either of two general approaches. First, polypeptides having fewer than about 100 amino acids, more usually fewer than about 80 amino acids, and typically fewer than about 50 amino acids, may be synthesized by the well-known Merrifield solid-phase synthesis method where amino acids are sequentially added to a growing chain (Merrifield (1963) J. Am. Chem. Soc., 85:2149-2156).

The second and preferred method for synthesizing the polypeptides of the present invention involves the expression in cultured cells of recombinant DNA molecules encoding a desired portion of the FIV genome. The portion of the FIV genome may itself be natural or synthetic, with natural genes obtainable from the isolated virus by conventional techniques. Of course, the genome of FIV is RNA, and it will be necessary to transcribe the natural RNA into DNA by conventional techniques employing reverse transcriptase. Alternatively, polynucleotides may be synthesized by well-known techniques. For example, short single-stranded DNA fragments may be prepared by the phosphoramidite method described by Beaucage and Carruthers (1981), *Tetrahedron Letters* 22:1859-1862. Double-stranded fragments may then be obtained either by synthesizing the complementary strand and then annealing the

strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

The natural or synthetic DNA fragments coding for the desired FIV protein or fragment may be incorporated in a DNA construct capable of introduction to and expression in *n* *vitro* cell culture. Usually, the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria. They may also be intended for introduction and integration within the genome of cultured mammalian or other eukaryotic cells. DNA constructs prepared for introduction into bacteria or yeast will include a replication system recognized by the host, the FIV DNA fragment encoding the desired polypeptide product, transcriptional and translational initiation regulatory sequences joined to the 5'-end of the FIV DNA fragment, and transcriptional and translational termination regulatory sequences joined to the 3'-end of the fragment. The transcriptional regulatory sequences will include a heterologous promoter which is recognized by the host. Conveniently, a variety of suitable expression vectors are commercially available for a number of hosts.

To be useful in the detection methods of the present invention, the polypeptides are obtained in a substantially pure form, that is, typically from about 50% W/W or more purity, substantially free of interfering proteins and contaminants. Preferably, the FIV polypeptides are isolated or synthesized in a purity of at least 80% W/W, and more preferably, in at least about 95% W/W purity. Using conventional protein purification techniques, homogeneous polypeptide compositions of at least about 99% W/W purity can be obtained. For example, the proteins may be purified by use of the antibodies described hereinafter using the immunoabsorbant affinity columns described hereinabove.

Once a sufficient quantity of natural or synthetic FIV polypeptides have been obtained, polyclonal antibodies specific for FIV may be produced by *n* *vitro* or *in vivo* techniques. *In vitro* techniques involved *in vitro* exposure lymphocytes to the antigenic polypeptides, while *in vivo* techniques require the injection of the polypeptides into a wide variety of vertebrates. Suitable vertebrates are non-human, including mice, rats, rabbits, sheep, goats, and the like. Polypeptides having more than about thirty amino acids, usually more than about fifty amino acids, may serve directly as the immunogen. If the polypeptide is smaller than about 10kD, particularly less than about 6kD, however, it may be necessary to join the polypeptide to a larger molecule to elicit the desired immune response. The immunogens are then injected into the animal according to a predetermined schedule, and the animals are bled periodically with successive bleeds having improved titer and specificity. Injections may be made intramuscularly, subcutaneously, or the like, and an adjuvant, such as a combination of complete and incomplete Freund's adjuvant, will usually be employed. The whole virus can also be used as the immunogen, although selection of antibodies specific for a particular determinant will be more difficult.

If desired, monoclonal antibodies can be obtained by preparing immortalized cell lines capable of producing antibodies having the desired specificity. Such immortalized cell lines may be produced in a variety of ways. Conveniently, a small vertebrate, such as a mouse, is hyperimmunized with the desired antigen by the method just described. The vertebrate is then killed,

usually several days after the final immunization, the spleen removed, and the spleen cells immortalized. The manner of immortalization is not critical. Presently, the most common technique is fusion with a myeloma cell fusion partner, as first described by Kohler and Milstein (1976) *Eur. J. Immunol.* 6:511-519. Other techniques include EBV transformation, transformation with oncogenes, retroviruses, etc., or any other method which provides for stable maintenance of the cell line and production of monoclonal antibodies.

When employing fusion with a fusion partner, the manner of fusion is not critical and various techniques may be employed. Conveniently, the spleen cells and myeloma cells are combined in the presence of a non-ionic detergent, usually polyethylene glycol, and other additives such as Dulbecco's Modified Eagle's medium, for a few minutes. At the end of the fusion, the non-ionic detergent is rapidly removed by washing the cells. The fused cells are promptly dispensed in small culture wells (usually in a microtiter plate at relatively low density, ranging from about one to  $5 \times 10^5$  cells/well), in a selective medium chosen to support growth of the hybrid cells while being lethal to the myeloma cells. Usually, the myeloma cell line has been mutated to be sensitive to a lethal agent, typically being HAT sensitive, and the medium includes a HAT concentration sufficient to inhibit the proliferation of the unfused myeloma cells.

After sufficient time, usually from about one to two weeks, colonies of hybrids are observed and plates containing hyperpositive wells are identified. The plates and wells having only one colony per well are selected, and supernatants from these wells are tested for binding activity against FIV or a particular FIV protein. Once positive hybridomas are identified, the cell line can be maintained as a viable culture and/or a quantity of the virus may be grown out, separated, and stored by lyophilization.

Depending on the desired use for the antibodies, further screening of the hybridomas may be desirable. For use in immunodiagnostic assays, antibodies having very high specificity and affinity for the antigenic site are desirable.

Once the desired hybridomas have been selected, monoclonal antibodies may be isolated from supernatants of the growing colonies. The yield of antibodies obtained however, is usually low. The yield may be enhanced by various techniques, such as injection of the hybridoma cell line into the peritoneal cavity of a vertebrate host. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Proteinaceous and other contaminants will usually be removed from the monoclonal antibodies prior to use by conventional techniques, e.g., chromatography, gel filtration, precipitation, extraction, or the like.

The polypeptides and antibodies of the present invention may be used with or without modification for the detection of or vaccination against FIV infection. Frequently, the polypeptides and antibodies will be labelled by joining, either covalently or non-covalently, a substance which provides for detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Some of the labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescers, chemiluminescers, magnetic particles and the like. Patents teaching the use of such labels include U.S.

Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Antibodies and polypeptides prepared as described above can be used in various immunological techniques for detecting FIV and anti-FIV antibodies in physiological specimens, particularly body fluid samples, including blood, plasma, serum, urine, and the like, and cell samples, such as lymphocytes. Depending on the nature of the sample, both immunoassays and immunohistochemical staining techniques may find use.

Liquid phase immunoassays and Western blot analysis will find use in detection of FIV in body fluids, particularly blood and urine. The use of antibodies in protein binding assays is well established. Numerous competitive and noncompetitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available. Detailed methods for detecting the presence of the viruses in serum samples are set forth in the Experimental section hereinafter. Additionally, enzyme linked immunosorbent assays (ELISA) for detecting presence of antibodies to FIV in blood are also set forth in the Experimental section.

Compositions of the present invention are also useful in preparing vaccines for protection against FIV infection. For example, the whole virus and/or FIV-infected cell lines may be wholly or partially inactivated and utilized as an immunogen in a vaccine composition. Partial inactivation may be achieved by passage at elevated temperatures or by contact with mutagens, such as ultraviolet light, ethyl methanesulfonate, and the like. Complete inactivation may be achieved by contact with other agents, including formalin, paraformaldehyde, phenol,  $\alpha$ -lactopropionate, ultraviolet light, heat, psorlens, platinum complexes, ozone and other viricidal agents.

Specific methods for the preparation of inactivated whole virus and FIV-infected cell line vaccines are described in detail in the Experimental section hereinafter. Conveniently, the source of whole FIV can be FIV-infected cell lines which have been found to be prolific producers, such as FL-4 and FL-6. Inactivated FL-4 and FL-6 are also suitable for preparing inactivated or attenuated whole cell vaccines.

The viral proteins and portions thereof, prepared as described above, may also be used in the preparation of subunit vaccines prepared by known techniques. Polypeptides displaying antigenic regions capable of eliciting protective immune response are selected and incorporated in an appropriate carrier. Alternatively, an antigenic portion of a viral protein or proteins may be incorporated into a larger protein by expression of fused proteins. The preparation of subunit vaccines for other viruses is described in various references, including Lerner et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:3403 and Bhattacharjee et al. (1982) Proc. Natl. Acad. Sci. U.S.A. 79:4400. See also, U.S. Pat. Nos. 4,565,697 (where a naturally-derived viral protein is incorporated into a vaccine composition); 4,528,217 and 4,575,495 (where synthetic peptides forming a portion of a viral protein are incorporated into a vaccine composition). Other methods for forming vaccines employing only a portion of the viral proteins are described in U.S. Pat. Nos. 4,552,757; 4,552,758; and 4,593,002. The relevant portions of each of these cited references and patents are incorporated herein by reference.

The vaccines prepared as described above may be administered in any conventional manner, including

oronasally, subcutaneously, intraperitoneally or intramuscularly, except that oronasal administration will usually not be employed with a partially inactivated virus vaccine. Adjuvants will also find use with subcutaneous and intramuscular injection of completely inactivated vaccines to enhance the immune response. The preparation of viral vaccine compositions optionally employing adjuvants is described in numerous standard references, such as Remington's *Pharmaceutical Sciences*, 10 Mack Publishing Co., Easton, Pa., 16th ed., 1982, the disclosure of which is incorporated herein by reference.

The dosage form and immunogen content of the vaccine will vary depending on the nature of the immunogen (i.e., whole virus, infected cell, or subunit) and the route of administration. Usually, a single dose will have a total volume including carrier, adjuvant, and any other components, in the range from about 0.1 ml to about 5 ml, more usually being from about 0.5 ml, more usually being from about 0.5 ml to about 3 ml. The amount of inactivated or attenuated whole FIV in each dose will usually be in the range from about 0.1 mg to about 5 mg, usually being from about 0.2 mg to 2 mg. For inactivated FIV-infected cell lines, each dose will typically contain from about  $10^6$  to  $10^8$  cells, usually about  $5 \times 10^6$  to  $5 \times 10^7$  cells.

The number and temporal spacings of the inoculations will be sufficient to elicit the desired immunoprotective response against subsequent challenge by FIV. Usually, there will be at least two inoculations spaced at least one week apart, more usually being from two to 10 inoculations spaced over a period from two to thirty weeks. Often, a final inoculation may be administered at some longer interval following an initial series of administrations. The selection of optimum administration patterns for a particular vaccine formulation is well within the skill in the art.

Diagnostic tests for detecting the presence of FIV in biological samples may also be performed using polynucleotide probes. Such polynucleotide probes may be prepared based on the sequence of the viral genome. The length of the probe is not critical, but will usually comprise at least about 12 bases, more usually comprising at least about 16 bases, which are substantially complementary to a portion of the viral genome. The probe itself may be DNA or RNA, and the probe need not have perfect complementarity with the FIV genome, with one or two mismatched pairs being acceptable for probes up to 20 bases in length and three to five mismatched pairs in probes from 20 to 35 bases. The probes may be prepared synthetically, with suitable synthetic techniques having been described above, and will include a detectable label. Usually, the synthetic sequences are expanded in commonly available cloning vectors and suitable hosts in order to obtain large quantities. The expanded vectors may themselves be labelled for use as probes, or shorter fragments containing complementary strands may be excised and labelled. Methods for the preparation and utilization of nucleotide probes for diagnostic testing are described in Falkow et al. U.S. Pat. No. 4,358,535, the disclosure of which is incorporated herein by reference.

A variety of labels have been employed, including those which have been described above for use in immunoassays, particularly radionuclides. Suitable labels may be bound to the probe by a variety of techniques. Commonly employed is nick translation with  $\alpha$ - $^{32}$ P-dNTP terminal phosphate hydrolysis with alkaline phosphatase followed by 5'-end labelling with radioac-

tive<sup>32</sup>P employing 7-P-NTP and T4 polynucleotide kinase or 3'-end labelling with an  $\alpha$ -<sup>32</sup>P-dNPT and terminal deoxynuoleotidyl transferase. Alternatively, nucleotides can be synthesized where one or more of the atoms present are replaced with a radioactive isotope, e.g., hydrogen with tritium. In addition, various linking groups can be employed. The terminal hydroxol can be esterified with inorganic acids, e.g., <sup>32</sup>P phosphate or <sup>14</sup>C organic acids, or else esterified with bifunctional reagents to provide other reactive groups to which labels can be linked.

The following examples are offered by way of illustration, not by way of limitation.

The experimental work described below relating to the development and use of cell lines FeT-1M, FeT-2D, FL-4, and FL-6 was performed in the laboratory of Dr. Janet K. Yamamoto at the University of California, Davis, California.

## EXPERIMENTAL

### Materials and Methods

#### Cell Types

Cells used as the source of FIV were the Crandell feline kidney cell line (FIV-CRFK) and feline mixed fresh PBLs (FIV-FeT1). Both cell types were infected with the Petaluma strain of FIV (A.T.C.C. No. VR 2186; deposited on Aug. 5, 1987, in connection with parent application Ser. No. 07/089,700). The FIV-CRFK line grows as a monolayer, morphologically similar to uninfected CRFK cells (Yamamoto et al. (1988) Am. J. Vet. Res. 49:1246-1258 and Fabricant et al. (1971) J. Am. Vet. Med. Assoc. 158:976-980). FIV-FeT1 cells, like uninfected FeT1 cells (mixed peripheral blood lymphocyte (PBL) cells from specific pathogen free (SPF) cats), grow in suspension and require interleukin-2 (IL-2). The IL-2-independent feline leukocyte cell lines, FL-4 and FL-6, were derived from the FIV-FeT1 cells and also are suspension cells which spontaneously produce FIV.

#### Cell Cultures

All suspension cell lines used in this study (FeT1, FL-4, FL-6) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES (N-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid), 2 mM L-glutamine, 50  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$ M 2-mercaptoethanol. IL-2-dependent cells were supplemented with 100 U/ml of recombinant human IL-2 (Cetus Corporation, Emeryville, Calif.). The suspension cells were passaged at a cell concentration of  $0.5-4 \times 10^6$  cells/ml and recultured in fresh culture media twice a week. FIV-CRFK cells were cultured in media consisting of equal volumes of L-15 and Eagle's minimum essential media, 10% heat-inactivated FCS, and 50  $\mu$ g/ml gentamicin. All monolayer cells were passaged twice a week at an initial cell concentration of  $2 \times 10^6$  cells/ml. The FIV-infected tissue culture fluids (TCF) were harvested twice a week, spun at 3000 rpm for 1 hr to remove residual cells, and stored at -20° C. or -70° C. or at 5° C. for those scheduled to be used within 1-5 days. One ml samples of cell-free infected TCF were routinely tested for Mg<sup>++</sup>-dependent reverse transcriptase (RT) activity as a means of monitoring for FIV production. Infected TCF were also checked routinely for Mn<sup>++</sup>-dependent RT activity to ensure that the cultures were producing only Mg<sup>++</sup>-dependent feline retrovirus (i.e., FIV). The RT assay used poly(rA)oligo(dT<sub>12-18</sub>) as an exogenous tem-

plate primer, four different deoxyribonucleotide triphosphates, 20 mM KCl with Mg<sup>++</sup> for detecting FIV or 60 mM NaCl with Mn<sup>++</sup> for detecting Mn<sup>++</sup>-dependent viruses (such as FeLV) and 5  $\mu$ Ci (<sup>3</sup>H)TTP alone per sample (Rey et al. (1984) Biophys. Res. Commun. 121:126-133). Five  $\mu$ Ci of (<sup>3</sup>H)TTP gave an average total count of 450,000 cpm using scintillation fluid mixture (1 part xylene to 2 part Amersham biodegradable counting scintillant) on a Bechman LS250 scintillation counter. As a result, our RT values will be below 450,000 cpm/ml.

### Development of IL-2-Independent FIV Producer Lines

IL-2-independent FIV producing cell lines were developed from an IL-2-dependent FIV-infected feline PBL line (FIV-FeT1). The process of gradual IL-2 depletion from the FIV-FeT1 cell line took extensive sub-culturing over a period of approximately three months. The depletion process entailed a gradual reduction of the percentage of IL-2 containing media from the culture in the following weekly sequence: 75%, 50%, 25%, 5% and 0% IL-2-containing media. During this period over 80% of the starting cultures which were depleted of IL-2 did not survive the procedure. Surviving cultures were placed in individual 2-cm<sup>2</sup> multiwells at a viable cell concentration of  $2 \times 10^6$  cells/ml/well. During this stage only three of starting 20 cultures survived and these cultures were expanded sequentially into 25-cm<sup>2</sup>, 75-cm<sup>2</sup>, and 175-cm<sup>2</sup> flasks. One of the cultures (FL-5) did not survive. RT assays were performed on the surviving two cultures (FL-4 and FL-6) during the expansion period as means of monitoring FIV production.

### In Vitro Infectivity Studies

The clarified infectious TCF from FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells was filtered individually with 0.45  $\mu$ m sterile filters to remove residual cells. These FIV inocula were aliquoted into 8-ml samples, stored at -70° C. and samples of these frozen inocula were retested for RT activity prior to in vitro infectivity studies. In all studies, the frozen inocula were thawed at room temperature immediately prior to use. FIV-susceptible feline cells ( $1 \times 10^6$  cells/ml) were infected with FIV at RT activity of 30,000 cpm/ml. All of the FIV-susceptible cells used in this study, with the exception of uninfected CRFK, were IL-2-dependent lymphoid cells which grew in suspension and required no trypsinization for passage. The TCF of the infected test cultures was harvested twice a week and the cells were recultured in fresh culture media containing IL-2. The harvested TCF was routinely tested for RT activity.

### In Vivo Infectivity Studies

Two specific pathogen free (SPF) cats, 11 months of age, were inoculated intraperitoneally (IP) with 2 ml of infectious TCF from either FL-4 or FL-6 cells. Infectious TCF from FL-4 or FL-6 cultures, having RT activities of 150,000 cpm/ml, was aliquoted and stored at -70° C. The frozen virus inocula were thawed at room temperature and filtered with 0.45  $\mu$ m Millipore filter just prior to the inoculation. Both the single freeze-thawing and the filtering procedure ensured that the inocula were free of viable cells. The cats were bled routinely to obtain serum for serological assays and PBLs for virus isolation. Virus isolation consisted of

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co-cultivating  $2-10 \times 10^5$  cells/ml with equal number of FIV-susceptible uninfected FeT1 cells and monitoring the TCF from these cultures for six weeks by RT assay. The PBL were considered positive for FIV isolation when RT activity of  $>10,000$  cpm/ml were detected in TCF from at least two consecutive harvest days. The RT activity of the TCF from co-culturing PBL from SPF cats with FeT1 cells was  $<2,500$  cpm/ml.

#### FIV Purification

FIV from infected TCF was concentrated and purified by ultracentrifugation, first on a 10/50% (w,v) discontinuous sucrose gradient and then on a 10/50% continuous sucrose gradient (Pedersen et al. (1987) Science 235:790-793 and Yamamoto et al. (1988) Leukemia, December Supplement 2:204S-215S). The virus purified by this procedure was used for comparing the biochemical properties of FIV derived from different culture preparations and as the viral substrate for the immunoblot assay. Immunoblot analyses of gradient purified FIV from different infected cell lines (FL-4, FL-6, FIV-FeT1, and FIV-CRFK cells) demonstrated the presence of the envelope gp100 band in blots from all viral sources. However, one major difference observed during these studies was that the intensity of the gp100 band was always weaker on the immunoblots made from purified FIV of FIV-CRFK origin than from those produced by other infected cell lines. Consequently, more viral antigen from FIV-CRFK cells was needed on the blots to get comparable intensity at the envelope band.

#### Immunoblot Analysis

A modification of the immunoblot technique described by Carlson et al. was used (Carlson et al. (1985) JAMA 253:3405-3408). Serum samples from immunized or FIV-infected cats were diluted to 1:50 in Buffer 3 (0.15M sodium chloride, 0.001M ethylene diaminetetraacetic acid, 0.05M Tris base, 0.05% Tween 20, and 0.1% bovine serum albumin) and incubated with the virus blot strips in individual wells for 18 hours at 37° C. These blot strips were then processed using a modification of a previously described procedure (Yamamoto et al. (1988) supra.). Briefly, the strips were incubated individually in wells with biotinylated anti-cat IgG (Vector Laboratories, Burlingame, Calif.) for 30 min and washed three times with wash solution. The strips were then incubated individually with horseradish peroxidase Avidin D (Vector Laboratories) for 30 min. After extensive washing, the strips were incubated with a fresh substrate solution (0.05% diaminobenzidine, 400 µg/ml NiCl<sub>2</sub> and 0.01% H<sub>2</sub>O<sub>2</sub> in 0.1M Tris buffer, pH 7.4) at room temperature. After establishment of visible bands the reactions were stopped with excess distilled H<sub>2</sub>O, and the strips were then dried.

#### FIV p28 Assay

The FIV core protein p28 was detected by an enzyme-linked immunoassorbent assay (ELISA) using two different monoclonal antibodies. A1 and B1 mAbs, to FIV p28 as either capture or substrate-reactive antibodies, respectively. Reactivity of both mAbs to FIV p28 antigen was confirmed by immunoblot analysis. The capture antibody (mAb A1) was coated on the plate overnight with bicarbonate buffer (pH 9.6) and washed once before its use. Serum samples to be tested were diluted in Buffer 3 and then incubated in the coated wells for 30 min at 37° C. The wells were

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washed six times with washing buffer, incubated with biotinylated mAb B1 for 30 min at 37° C., washed six times more, and then incubated with horseradish peroxidase Avidin D for 15 min. The wells were washed extensively again and finally incubated with substrate solution (0.005% tetramethylbenzidine and 0.015% H<sub>2</sub>O<sub>2</sub> in 0.96% citric acid solution) at room temperature. The reactions were stopped with 1M sulfuric acid solution upon establishment of a visible color reaction in the sequentially diluted standards consisting of purified FIV from pooled FIV-CRFK and FIV-FeT1 preparations.

#### Characterization of FL-4 and FL-6 Cell Lines

The phenotypic profiles of the feline cells were determined by fluorescence activated cell sorter (FACS) analysis using characterized monoclonal antibodies to feline CD4 (Fel 7), CD8 (FT2), pan T-cell, and to feline light chain and µ heavy chain specific (AC5) markers (Ackley et al. (1990) supra.; Ackley et al. (1990) supra.; Klotz et al. (1986) J. Immunol. 136:2510-2516; and Klotz et al. (1985) J. Immunol. 134:95-99). The cells were tested for mycoplasma using two different procedures both performed by Bionique Laboratories, Inc. The first procedure consisted of the direct DNA-fluorochrome staining of the cells for mycoplasma. The second procedure involved passaging test cells onto indicator cells which were then DNA/fluorochrome stained for mycoplasma. Detection of FeLV p27 core antigen was performed using the p27 antigen ELISA assay (Lutz et al. (1983) J. Immunol. Methods 56:209-220). Polymerase chain reaction (PCR) was used to test for the presence of FeLV provirus DNA. Briefly, a pair of primer sequences from the U3 region of the FeLV LTR were chosen so as to avoid the possibility of overlap with endogenous sequences of FeLV. The sequences of the two oligonucleotides primers used for PCR were 14 base pairs (bp 24 to 37) and 17 base pairs (bp 239 to 255) long. This enabled us to amplify a sequence of 232 base pairs for which we prepared a 25 base pair probe (bp 203 to 227) labeled with <sup>32</sup>P for identification by Southern blotting. The indirect fluorescent antibody assay to detect feline syncytium-forming virus (FeSFV) was performed as described previously (Pedersen et al. (1987) supra.; Yamamoto et al. (1988) supra.; and Yamamoto et al. (1989) J. Am. Vet. Med. Assoc. 194:213-220).

#### Immunogenicity of FIV Produced from FL-4 and FL-6 Cells

Eighteen SPF cats, 4-6 months of age, were used in these studies. Some of these cats were previously exposed to feline herpes virus (FHV)(A.T.C.C. C-27 strain) and the cats were free of FHV symptoms two-weeks prior to and during immunization. Three of the cats were immunized four times with 200 µg of inactivated FIV (inactivated whole virus) particles that were produced by pelleting cell-free TCF of FL-4 cells. An additional three cats were immunized eight times with 20 µm of inactivated whole virus. Seven cats were immunized either four or six times with  $1 \times 10^7$  cells per dose of inactivated FL-6 or FL-4 cells (inactivated whole cell-virus), respectively. The pelleted virus and the infected cells were each inactivated with 1.25% paraformaldehyde, dialyzed against PBS, and then combined with adjuvant just prior to immunization. The adjuvants used were either threonyl muramyl dipeptide (MDP) (Byars et al. (1987) Vaccine 5:223-228)

or a combination of Freund's complete and incomplete adjuvants. Control cats were immunized with either uninfected FeT1 cells with adjuvant or diluent with adjuvant. All cats were immunized at two week intervals for a total of four or eight immunizations, unless stated otherwise.

## RESULTS

### Development of IL-2-Independent FIV-Producing Cell Lines

The development of IL-2-independent cell lines from FIV infected mixed PBLs (FIV-FeT1 cells) entailed the gradual depletion of IL-2 from the cultures. Only two out of 20 cultures, FL-4 and FL-6, survived the depletion process. Significant RT titers (100,000–400,000 cpm/ml), Mg<sup>++</sup>-cation-dependent, were detected in these cultures during the expansion and large scale-production stage. Electron microscopy demonstrated numerous typical lentivirus particles in these cultures (data not shown).

The growth rates of these cell lines were compared to those of FIV-FeT1 and FIV-CRFK. The viable cell doubling time for FL-6 was found to be approximately 24 hours, whereas the doubling time for FL-6 was found to be approximately 24 hrs, whereas the doubling time for FL-4 was approximately 48 hrs. Both cell lines grew at an exponential rate. From a starting cell concentration of  $5 \times 10^5$  cells/ml, peak viable cell counts were observed after 3–4 days of culturing. Viability of the cells present in these cultures ranged from 70 to 90% over the four day culturing period. The number of syncytial cells in the FL-4 and FL-6 cultures was less than 0.1%. In comparison, the viability of FIV-FeT1 cells was only 55 to 65% after 1 day of culture, which may be attributed to dependence on IL-2. In our hands, a majority of the IL-2-dependent feline (FeT1, FeT2) and murine (HT-2C, CTLL-2) lymphoid cell lines have similar viability profiles. In order to evaluate the correlation between cell growth and virus production, samples from different harvest days were assayed for RT activity (FIG. 1). At a starting cell concentration of  $5 \times 10^5$  cells/ml, peak RT titers were observed on Day 4 of culture. Based on the number of cells present on Day 4, FL-4 cells produced the highest and FIV-CRFK the lowest RT activity.

### Characterization of the FL-4 and FL-6 Cell Lines

The phenotypic profiles of FL-4 and FL-6 cells were determined by flow cytometric analysis using monoclonal antibodies (mAb) to feline CD4 (fel 7), CD8 (FT2), pan T-cell (42) markers (Ackley et al. (1990) J. Virol. 64:5652–5655; Carlson et al. (1985) supra.; Ackley et al. (1990) supra.; and Klotz et al. (1986) supra.) and mAb that detect both feline immunoglobulin light chains and  $\mu$  heavy chain (ACS) (Klotz et al. (1985) supra.). The FACS profiles demonstrated that FL-4 cells were CD4 $\pm$ , CD8 $+$ , and Pan-T $+$  whereas FL-6 cells were CD4 $-$ , CD8 $\pm$ , and Pan-T $+$ . Both cell lines were negative for surface IgM and  $\lambda$  and  $\kappa$  light chains. It should be noted that both CD4 and CD8 antigens were lost in cultures maintained for several months. FL-4 and FL-6 cells were >95% positive by IFA for surface FIV antigen expression using polyclonal antibodies to FIV (Table 1). Additional tests were performed to ensure that these cells were free of known contaminants which could limit their use. The results are summarized in Table 1. The two cell lines were mycoplasma-free both by direct DNA/fluoro-

chrome stain and indirectly by passaging onto indicator cells prior to staining. Furthermore, FL-4 and FL-6 cells were shown to be negative for FeLV core protein p27 expression by ELISA and for exogenous FeLV DNA by PCR. The cells were determined to be negative by IFA for feline syncytial-forming virus (FeSFV).

TABLE 1

Absence of Known Contaminants in FL-4 and FL-6 Cell Lines			
Micro-organism Tested	Infection Status	Antigen Detected	Method of Testing
FIV	+	viral RT <sup>a</sup>	Mg <sup>++</sup> -dependent RT
	+	viral antigens <sup>ab</sup>	Immunoblot analysis with FIV-positive serum.
	+	mature virion	Electron microscopy.
	+	whole cell	IFA with FIV-positive serum (>95% positive).
FeLV	–	viral RT <sup>a</sup>	Mn <sup>++</sup> -dependent RT.
	–	viral core	ELISA.
	–	p27	
	–	mature virion proviral LTR sequence in cellular DNA	Electron microscopy. PCR and Southern blot.
FeSFV	–	mature virion	Electron microscopy.
	–	whole cell	IFA with FeSFV-positive serum.
Mycoplasma	–	whole cell	Direct DNA/fluorochrome staining for mycoplasma. Indirectly by staining indicator cells which were passaged with FL-4 and FL-6 cells.

<sup>a</sup>These tests were performed on the tissue culture fluid harvested from the FL-4 and FL-6 cell cultures.

<sup>b</sup>Purified virus was disrupted with 0.1% SDS prior to its use in immunoblot production, as described in Methods.

### FIV Production in FL-4 and FL-6 Cell Lines

The amount of FIV produced from the FL-4, FL-6, FIV-FeT1 and FIV-CRFK cell lines was determined by comparing the total protein and RT levels of FIV in different fractions from sucrose gradient preparations (data not shown). High titers of both RT activity and total protein were observed in FIV preparations from FL-4, FL-6, and FIV-FeT1 cells. The FIV-CRFK produced low titers of FIV as demonstrated by the low levels of both protein concentration and RT activity in the fractions. The three peak fractions of the gradient purified virus from each cell line were pooled and measured for total protein concentration, RT titer, and viral core protein (p28) concentration. The results demonstrated a direct correlation between the p28, RT and the total protein levels present in the purified virus preparations. The viral antigen profiles of the FIV produced by the various cell lines were also compared by immunoblot analysis. Different concentrations of purified virus from different cell sources were used as substrate antigen for immunoblot strips. These immunoblots were then reacted with a set concentration of FIV-seropositive cat sera and the banding patterns evaluated. The immunoblot profiles from FL-4 and FL-6 cells were similar to those of FIV-FeT1 and FIV-CRFK (data not shown). The intensity of the bands, especially the viral envelope and transmembrane glycoproteins, produced on immunoblots from FL-4, FL-6 and FIV-FeT1 viral

substrates was stronger than that from FIV-CRFK. In addition, immunoblots of significantly higher quality were produced from the larger quantity of purified virus obtained from the TCF of FL-4 and FL-6. Thus, these results further indicate that a larger amount of virus antigens was produced by the FL-4 and FL-6 cell lines than by the FIV-FeT1 and FIV-CRFK.

#### Characterization of FIV Produced From FL-4 and FL-6 Cells

The FIV produced from FL-4 and FL-6 cells was tested for its ability to infect FIV-susceptible cell lines (FIG. 3). Cell-free TCF from different infected cell lines was inoculated into various feline cell cultures at a set RT concentration of 30,000 cpm/ml. FIV from FIV-CRFK cells did not readily infect certain feline lymphoid cells, in particular thymus-derived cultures, as compared to the FIV from FL-4 and FIV-FeT1 cells. The FIV from FL-6 cells was also highly infectious to FIV-susceptible cell lines (data not shown). Next, the FIV preparations produced from FL-4 and FL-6 cells were tested for their ability to infect SPF cats (FIG. 4). One SPF cat each was inoculated IP with 2 ml of cell-free TCF from either FL-4 or FL-6 cells. Both cats developed antibodies to FIV within four weeks post-infection. By sixteen weeks post-infection, the immunoblot profiles of these sera demonstrated the presence of antibodies to the majority of viral core antigens, but not to the viral envelope or transmembrane glycoproteins. Both cats were positive for virus isolation from PBLs. These studies demonstrated that the virus preparations from the FL-4 and FL-6 cell lines were highly infectious in both *in vitro* and *in vivo* systems.

#### Immunogenicity of FIV Produced From FL-4 and FL-6 Cells

Immunization of four cats with the inactivated FL-4 cell preparations ( $1 \times 10^7$  cells) led to the production of FIV antibodies specific for the viral core protein p28 soon after the second immunization (FIG. 5A). Antibodies to other viral antigens were demonstrated only after the third or fourth immunization (FIG. 5A). Thus the development of the antibodies in immunized cats closely mimics the FIV antibody development in experimentally infected cats (Yamamoto et al. (1988) *supra*, and Hosie et al. (1990) *supra*). Control cats immunized with uninfected FeT1 cell preparations did not develop viral antibodies over the duration of the six immunizations.

Six other cats were immunized with inactivated FL-4-produced virus (200 µg) or inactivated FL-6 cell ( $1 \times 10^7$  cells) preparations together with a combination of complete and incomplete Freund's adjuvant instead of MDP (FIG. 5B). Both inocula led to the production of antibodies specific to the viral p28 shortly after the second immunization. Two out of the three cats immunized with the inactivated virus preparation developed antibody responses to viral envelope, whereas all three cats immunized with the inactivated FL-6 cell preparation developed antibodies to the envelope shortly after the second immunization. When other cats were immunized with 20 µg of pelleted inactivated virus in MDP) per dose, two out of three cats developed antibodies to the viral envelope, but only after the sixth immunization (FIG. 5B). Furthermore,  $1 \times 10^7$  viable FL-4 or FL-6 cells released into the TCF approximately 10 µg equivalence of purified virus (by sucrose gradient method) or approximately 30 µg equivalence of crude pelleted

virus when at their peak production level (data not shown). These findings suggest that it is more practical to use inactivated whole infected cells as an immunogen than inactivated whole virus for development of antibodies to the viral envelope. No difference in antibody development to viral p28 was observed between the infected cell or cell-free virus immunogens. Thus, our studies demonstrate that cats immunized with inactivated whole FIV-infected cells generate higher FIV envelope antibody titers more rapidly and more consistently than those immunized with inactivated cell-free whole virus.

#### Vaccination with Inactivated Cell Lines and Post Vaccination Challenge

The fixed cell-virus vaccine consisted of FIV-FeT1 and FIV-FL-4 inactivated with paraformaldehyde. In each culture (which was subsequently inactivated) essentially 100% of the cells were productively infected with FIV and  $5 \times 10^7$  cells were required to obtain  $100\mu$  of total viral protein. Analysis of the FIV-infected cells in both T-cell lines by immunoblot using serum from an FIV immunized cat and by Coomassie stain, showed that the vaccine preparations contained the env, gag and pol virion proteins and their precursors as well as some regulatory proteins and cellular proteins (data not shown). The adjuvant used was threonylmuramyl dipeptide (MDP) (Syntex SAF-A).

The infected cells were inactivated with 1.25% paraformaldehyde for 24 hrs and washed three times with phosphate buffered saline (PBS). The vaccine consisted of  $1 \times 10^7$  inactivated FIV-FeT1 cells (Group 1A) or FIV-FL-4 cells (Group 1B) mixed with 250 µg of MDP. All cats in Group 1 were specific pathogen free (SPF) cats of 4-6 months of age, which were previously exposed to feline herpes virus (FHV C-27 strain) and were free of FHV symptoms two weeks prior to and during immunization. Ten control cats were immunized with either uninfected FeT1 cells with MDP (Group 1C) or MDP alone (Group 1D). All cats were challenged IP with 10 animal infectious doses (AID<sub>100</sub>) of homologous FIV petaluma strain two weeks after the final immunization. For the whole-virus vaccine, FIV was pelleted from the culture fluid of FIV-FL-4 cells, inactivated with 1.25% paraformaldehyde for 24 hours, and dialyzed extensively against PBS. Cats in Group 2A were SPF cats which were immunized with the inactivated FIV particles Group 2B cats received  $1 \times 10^7$  inactivated uninfected FeT1 cells mixed with the inactivated virus. Three additional SPF cats were immunized with either adjuvant (Cat #182) or diluent (Cats #55D and #55H). All cats were challenged with 10 ID FIV (homologous strain) two weeks after the final immunization. Virus was isolated from PBL and bone marrow cells by co-culturing with FIV-susceptible FeT1 cells. PCR analysis was performed using the method previously described (Pedersen et al. (1989) *J. Virol.* 64:598-606).

Nine cats in total were vaccinated subcutaneously (SC) with  $1 \times 10^7$  cells mixed with MDP (250 µg) five times at two week intervals and a final boost was given two months later. Five cats received the FIV-FeT1 cells (Group 1A) and four cats received the FIV-FL-4 cells (Group B). Ten control cats were inoculated with the uninfected allogeneic T-cells mixed with adjuvant, or adjuvant alone (Groups 1C and 1D, Table 2). Both fixed cell-virus vaccines induced significant levels of FIV antibodies (1:5,000 to 1:50,000) after the first-boost

as detected by whole-virus ELISA (FIG. 6A). By immunoblot, all nine vaccinated cats showed antibody to the viral core protein p24 after the first boost and antibody to other viral antigens, including the envelope gp100, after the third or fourth immunization (FIG. 7). Antibody response to the cellular components of the vaccine was weak as determined by immunoblot reactivity to the fixed uninfected cells. FIV-neutralizing antibody titers of 1:800 to 1:1200 were reached after the final immunization; these titers were equal to or slightly higher than the titers observed in unvaccinated cats experimentally infected with FIV (FIG. 8A). No infectious virus was detected in the vaccinated cats prior to challenge. Control cats remained free of antiviral antibodies and of infectious virus during the immunization schedule. The results are summarized in Table 2.

Virus was recovered persistently after 5 weeks pc from the PBLs of one vaccinated cat (#178) and after 21 weeks pc from another vaccinated cat (#138). The PBLs of both animals were PCR positive at 21 weeks pc at which time infectious virus was isolated from their bone marrow. These two persistently infected cats showed a sudden rise in antibodies by ELISA at the time virus was recovered, and the antibodies remained high thereafter (FIG. 6A). Also, by immunoblot, both core and envelope antibodies persisted longer in these cats than in the protected cats. These late breakthroughs of infectious virus in cats that had previously appeared protected indicate that only time can confirm the absence of latent virus in the protected vaccines. At 27 weeks pc, the seven vaccine protected cats showed FIV specific cell mediated response (CMR) as

TABLE 2

Isolation of FIV from Vaccinated Cats Before and After FIV-challenge								
GROUP 1A		GROUP 1B		GROUP 1C		GROUP CODE		
	CAT #				CAT #	GROUP 2A	GROUP 2B	GROUP 2C
135,137,142, 150,209	145,224,138, 178	136,147,164, 214,227		175,215,270, 271,278	55B,55F,094	55C,55I,177	182,55D,55H	
VACCINE TYPE								
FIV-FeT1 Cells	FIV-FL-4 Cells	FeT1 Cells (uninfected)		Placebo	Whole Virus	Whole Virus + FeT1 Cells (uninfected)		Placebo
<b>FIV ISOLATION (Positive Cat #) From PBL</b>								
Pre-challenge	—	—	—	—	ND	—	—	ND
2 weeks post- challenge (pc)	—	—	—	—	ND	ND	ND	ND
3 weeks pc	—	—	147,214,227	175,270,278	—	—	—	—
5 weeks pc	209	178	147,214,227	175,270,278	ND	ND	ND	ND
7 weeks pc	—	178	ALL	ALL	—	SSI	ALL	ALL
17 weeks pc	—	178	ALL	ALL	—	—	—	ALL
21 weeks pc	—	138,178	ALL	ALL	ND	ND	ND	ND
26 weeks pc	—	138,178	ALL	ALL	ND	ND	ND	ND
<b>From Bone Marrow</b>								
21 weeks pc	—	138,178	ALL	ALL	—	—	—	ALL
<b>PCR OF PBL</b>								
21 weeks pc	—	138,178	ALL	ALL	—	—	—	ALL
<b>TOTAL # INFECTED</b>	1/5	2/4	5/5	5/5	0/3	1/3	3/3	
<b>TOTAL # CHALLENGED</b>	(Transient)	(Persistent)	(Persistent)	(Persistent)		(Transient)		

— Indicates negative result.

Number indicates positive result from a specific cat with corresponding identification number.

ALL indicates that all cats in the specific group are positive.

ND indicates not done.

Two weeks after the final immunization, all of the cats were challenged intraperitoneally (IP) with 10 animal infectious doses (AID<sub>100</sub>) of the homologous FIV strain. Starting at seven weeks post challenge (pc) all ten control cats seroconverted, antibody titers gradually increased, and virus was persistently isolated from their peripheral blood lymphocytes (PBLs) (Table 2). By contrast, a steady fall in antibodies occurred and virus could not be isolated from the PBLs of six of the nine vaccinated cats for ≥21 weeks pc. These six cats were also free of detectable virus at 17 weeks pc as measured by bone marrow culture and polymerase chain reaction (PCR) analysis of PBLs and bone marrow cells. They therefore seemed to be solidly protected without evidence of latent proviral DNA. In one of the vaccinated cats (#209), virus was recovered from PBLs only one occasion, at 5 weeks pc, after which it was no longer detectable in either the PBLs (by virus isolation and PCR) or the bone marrow cells (by virus isolation). Antibody levels decreased steadily in this animal. Therefore, this animal may also be protected.

measured by positive lymphocyte proliferation and IL-2 induction assays (FIGS. 9A and 9B) as well as a positive response to non-specific mitogens (data not shown). By contrast, the two persistently infected vaccines and all infected control cats showed a lack of cellular response to FIV while the non-specific mitogen response remained intact. Since these cats were not tested for CMR before challenge we do not know if they were genetically poor responders and therefore vulnerable to infection or whether these defects in CMI were the result of infection. These findings suggest that the fixed cell-virus vaccine had induced T-cell immunity in the apparently protected cats. The duration of memory T-cell and the possible vaccine induction of cytotoxic T-lymphocytes remain to be determined.

#### 65 Vaccination with Inactivated Whole Virus and Post 66 Vaccination Challenge

The cell-free whole virus vaccine was prepared from FIV-FL-4. Virus released from this cell line in high titer

( $5 \times 10^8$  cells produced 1 mg viral protein per litter) was pelleted, filtered ( $0.45 \mu\text{m}$ ), inactivated with paraformaldehyde, and given with a combination of Freund's complete and incomplete adjuvants. Analysis of the cell-free pelleted FIV preparation from the whole-virus vaccine by immunoblot using serum from an FIV immunized cat showed that this vaccine contained all of the viral antigens, although a lesser amount of env glycoproteins than was present in the fixed infected cell vaccine, and also a trace amount of cellular antigens (data not shown). Six cats were immunized SC with 200  $\mu\text{g}$  viral proteins given every two weeks  $\times 4$ . Three control cats received either adjuvant or diluent alone. Significant levels of FIV antibodies (1:20,000 to 1:35,000) detected by whole-virus ELISA were induced after the first boost (FIG. 6B). By immunoblot, core p24 antibodies developed in all cats after the first boost and envelope gp100 antibodies developed in five of six cats after the third immunization (FIG. 7). Just prior to challenge, neutralizing antibody titers to FIV of 1:100 to 1:600 were present in all vaccinees (FIG. 8B). All cats were free of infectious virus prior to challenge.

Two weeks after the final immunization, cats were challenged IP with 10 ID of FIV. PBL cultures became virus and PCR positive by seven weeks pc from the three controls (Table 2), whereas five of six vaccinated cats remained uninfected up to 14 weeks. The PBLs of one vaccinated cat (#55I) were transiently infected at 7 weeks pc but were negative by virus isolation and PCR at 17 weeks pc. After challenge, gradual decreases in antibody titers were observed in all immunized and protected cats including the single transiently infected cat. These findings show that effective protection against FIV challenge infection can also be achieved with an inactivated whole-virus vaccine.

Both the fixed cell-virus and whole-virus vaccines appeared to give roughly equal protection against challenge infection. However, the whole-virus vaccine was less immunogenic and required about 20-fold more cells to produce the amount of viral protein required to raise antibody titers to the levels approximating those observed with the fixed cell-virus vaccine. Nevertheless, the neutralization titers were still several fold less with the cell-free virus vaccine. This difference can probably be attributed to the difference in adjuvant used and/or to the greater amount and integrity of viral antigens presented on infected cells as compared to cell-free virus (Yamamoto et al. (in the press) Intervirol., and Hosie and Jarrett (1990) AIDS 4:215-220). The cell-

virus vaccine may also have elicited an allogenic effect from the inclusion of other cellular antigens. However, a mixture of uninfected allogeneic (FeT1) cells and inactivated whole virus (Table 2, Group 2B) did not enhance the ELISA and neutralizing antibodies to FIV as compared to whole virus alone. This indicates that the expression of viral antigens on the infected cell apparently provides the most effective immunogenicity.

The specific viral proteins and specific immune responses that account for the vaccine protection observed are as yet uncertain. Viral envelope appears an essential determinant because, in another trial, cats immunized with an FIV Iscom vaccine that was deficient in envelope antigen failed to make gp120 antibody and were not protected against challenge infection with 20 ID<sub>50</sub> of homologous virus. The vaccines of the present invention probably achieved a minimal threshold of protection because, using a similar fixed cell-virus vaccine we were previously unable to protect against a higher challenge dose ( $5 \times 10^3$  ID) of virus (data not shown). Although neutralizing antibody would seem a logical mechanism, other means of vaccine protection, such as antibody dependent complement lysis or cellular cytotoxicity (ADCC) against cell-free virus or infected cells, may also contribute.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A vaccine against feline immunodeficiency virus infection comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, wherein said immunogen elicits an immune response protective against infection by FIV when administered to a susceptible host in an amount effective to elicit such response.

2. A method for protecting a susceptible cat against feline immunodeficiency virus infection, said method comprising administering to said cat a vaccine comprising an immunogen selected from the group consisting of inactivated whole FIV and an inactivated FIV-expressing cell line, in an amount effective to elicit an immune response protective against infection by FIV.

3. A vaccine composition as in claim 1, further comprising an adjuvant.

\* \* \* \* \*

ATTACHMENT D



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Page**Maintenance Fee Statement****5275813**

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 11, "STAT" below.

If a maintenance fee payment is defective, the reason is indicated by code in column 11, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,275,813	184	1950		07/739,014	01/04/94	07/31/91	08	NO	PAID

ITEM NBR	ATTY DKT NUMBER
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2307U-237-3/

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**BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV  
(1A55.20)**

Date	Description
28-Aug-91	Submitted FIV KV license application.
28-Aug-91	Submitted new FIV KV Production Outline.
28-Aug-91	Submitted research report entitled "Feline Immunodeficiency Virus Vaccine: Immunogenicity Study Proposal for USDA Licensure".
15-Nov-91	USDA approved the Production Outline with comments & pen-and-ink changes.
3-Feb-92	USDA responded to research report/protocol stating they are not yet prepared to state that the proposed study can meet the intended goal.
15-Jun-99	Requested USDA to transfer this product to their inactive files as we are discontinuing our efforts to license the product. The 2 strain product had, to this point, contained a strain (FIV alternate subtype A infected cell line) which we decided in 1999 to replace with the present strain (FIV subtype D infected cell line). The FIV subtype A infected cell line strain in the discontinued project remains in the present product due to be licensed (15A5.21). Although the 1A55.20 licensing project was cancelled in 1999, its FIV subtype A infected cell line fraction is actually the basis on which our 2001 license for 15A5.21 will be built.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
4-May-98	Submitted FIV-KV license application.
1-May-98	Submitted new FIV-KV Production Outline.
4-May-98	Submitted FIV-KV subtype A infected cell line master cell stock qualification report (MCS & MCS+25) and 2008s. Requested TA# to submit to CVB-L and permission to transfer to production.
4-May-98	Submitted FIV-KV alternate subtype A cell line master cell stock qualification report (MCS & MCS+25). Requested TA# to submit to CVB-L and permission to transfer to Production.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the subtype A infected cell line to Production at our own risk.
2-Jul-98	Received verbal (7/2/98) from Dr. Elsken to transfer the alternate subtype A cell line MCS to Production at our own risk.
13-Aug-98	Submitted efficacy protocol.
5-Nov-98	The license application has been filed.
1-Dec-98	The new Outline was approved w/ pen-and-ink changes & comments.
7-Jun-99	Letter (faxed) requesting permission to move virus fluid from R & D building to production room 520, bldg 115 to be killed by autoclaving.
9-Jun-99	Permission granted to move the virus fluid from R&D to Production. However, questions were raised with other CVB-IC personnel
2-Jul-99	Submitted letter for master seed/master cell stock qualification report for FIV subtype D infected cell line. Requested permission to submit cell line to CVB-L and transfer to our production department. Requested TA #.
15-Jul-99	7/15/99 received verbal permission to transfer FIV subtype D infected cell line to Production at our own risk & TA#7610 to submit MCS samples to CVB-L. Confirmation letter received on 7/23/99.
20-Jul-99	Submitted FIV subtype D infected cell line along with the X+25 to CVB-L for confirmatory testing.
20-Jul-99	Submitted subtype A infected cell line to CVB-L for confirmatory testing.
4-Aug-99	Responded to Renee's June 9, 1999, questions (approval was granted) in regard to our moving a bioreactor to Room 510 Building 115 for the purpose of autoclaving.
4-Aug-99	No response required to the questions in regard to moving a bioreactor to Room 510.
27-Aug-99	Submitted subtype A infected cell line samples for testing.
4-Oct-99	A revised efficacy protocol which includes sufficient detail to address USDA comments needs to be submitted for consideration by the CRT prior to initiation of the study.
12-Oct-99	Submitted a revised efficacy protocol in response to USDA's 10/4/99 letter prior to initiation of the study.
29-Dec-99	Approved the revised efficacy protocol for the purpose intended.
24-Jan-00	Authorization to ship inactivated FIV vaccines to Japan was not granted based on the info.i.e. VS Code ?, serial numbers.
14-Jan-00	Submitted letter to Dr. Carr requesting permission to ship two experimental inactivated FIV vaccines to Japan.
14-Feb-00	Submitted complete revision of Production Outline in response to USDA comments.
13-Mar-00	Submitted response to 1/24/00 letter regarding permission to ship FIV to Japan. Need to clarify if there is a VS Code, serial number, etc.
21-Mar-00	Approved the request to ship FIV inactivated vaccines to Japan with comments.
17-Apr-00	Submitted new FIV post inactivation virus testing Special Outline 217.
19-Apr-00	SO 217 returned unprocessed.
3-Jan-01	Submitted letter requesting permission to ship experimental vaccines of FIV & FIV/FeLV to Japan.
9-Jan-01	Authorization received to ship experimental products of FIV & FIV/FeLV to Japan.
16-Jan-01	Submitted new FIV post inactivation testing SO responding to USDA 2015 comments dated 4/19/00.
15-May-01	Submitted preliminary field safety trial protocol.
17-May-01	Submitted Immuno report & included a disk with statistical analysis.
17-May-01	Approved subtype A MCS X+25 infected cell line for use in production.
17-May-01	Approved subtype A infected cell line for use in production.
17-May-01	Approved the FIV subtype D infected cell line MCS & X+25 for use in production.
19-Jun-01	SO was approved w/ pen-and-ink changes and comments.
19-Jun-01	Submitted in-vitro report for FIV.
19-Jun-01	Approved the preliminary field trial protocol w/comments. A form for owners with adverse events/daily observations column with clear instructions. Our final report should state number of properly completed forms returned to cooperating veterinarians.
19-Jun-01	Approved the complete revision of the Outline (in response to USDA comments 2/14/00) with comments and pen-and-ink changes.
21-Jun-01	Obtained field trial authorization from state vets.
25-Jun-01	Submitted revised outline of production.
26-Jun-01	Submitted new SO 237 for ELISA potency test for FIV.
5-Jul-01	No response required to comments re: owners completing forms during field trial.
5-Jul-01	Submitted letter responding to comment re: owners completing forms during field trial study.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
16-Jul-01	Submitted request for field safety test w/ 2008s for 2 of the 3 prelicensing serials. (Notify AR, VA, MD, CA of any adverse reactions—see state letters for their individual request.)
23-Jul-01	State vet approvals received.
24-Jul-01	Submitted letter advising of corrected address for Missouri site (Dr. Roger Siferman) and copies of state approvals.
25-Jul-01	7/16/01 2008s for Serials 129050A & 129051A are filed as satisfactory. Verbal authorization on 7/20/01 to initiate field safety trial and ship 2000 doses of each serial. Amended protocol is approved.
25-Jul-01	USDA responded to the 7/5/01 ltr re: owners completing forms & their response was daily observation records from cat owners will not be required. Field safety protocol is adequate.
25-Jul-01	The change in address for the Missouri site was noted by USDA.
22-Aug-01	Immuno report filed as satisfactory.
30-Aug-01	Submitted ltr to Dr. Ludemann re: additional investigator for state of CA.
4-Sep-01	Verbal approval to ship to the additional investigator for the state of CA.
20-Sep-01	Submitted 2008 for 3rd PLS and requested TA#.
24-Sep-01	Submitted documentation of the storage temp of the Reference vaccine to support a 5 yr dating for frozen References. Approval of serial 1475-07-090299 (full dose) is contingent upon documentation (ltr of 8/22/01).
2-Oct-01	Received verbal TA#8279 to submit samples of the 3 PLS.
2-Oct-01	USDA approved new SO 237 ELISA potency.
3-Oct-01	USDA approved the revised Production Outline w/ comments & pen-and-ink changes.
5-Oct-01	Submitted samples of the 3 PLS to CVB-L under TA#8279 for confirmatory testing.
15-Oct-01	Reference 1475-07-090299 is approved for use as a Reference vaccine for serial release testing. The in vitro report satisfies concerns re: storage conditions of the immuno/ref serial. Need to submit FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 and approval of this report is contingent upon review of the subtype A infected cell line reaction w/ 1D9.
31-Oct-01	Submitted data for FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9.
2-Nov-01	The data submitted supporting the FIV subtype A infected cell line reaction w/ monoclonal antibody 1D9 was approved.
14-Nov-01	Submitted new label(s) (Spanish/English).
19-Nov-01	Submitted field trial report.
30-Nov-01	Changed FIV placebo vaccine lot# to 1516-62-031501. This lot was used in potency test validation and for all vaccine testing.
30-Nov-01	Submitted inactivation kinetics demonstrating that one inactivation procedure is sufficient for this virus per USDA 2015 comments dated 10/3/01.
4-Dec-01	Submitted "Supplemental Data - Addendum to the Demonstration of the Safety of FDAH's FIV Vaccine, KV in Cats Under Field Conditions". This should complete our submission for the field trial.
13-Dec-01	Submitted complete revision of SO 217.
18-Dec-01	Submitted results of study of experimental vaccines sent to Japan to CVB-L.
27-Dec-01	USDA approved the FIV placebo vaccine lot change subt'd on 11/30/01.
7-Jan-02	USDA approved the field trial report.
8-Jan-02	USDA approved the study of experimental vaccines sent to Japan.
9-Jan-02	USDA approved the inactivation kinetics.
9-Jan-02	CVB-L has completed satisfactory confirmatory testing of Prelicensing Serials 129050A, 129051A & 129052A.
4-Feb-02	Submitted new labels (FDAH requests the 10/22/01 & 11/14/01 submission be returned as sketches).
6-Feb-02	USDA approved labels submitted on 11/14/01 as sketches (per FDAH's request on 2/4/02).
21-Feb-02	Submitted new labels that replace sketches (FDAH requests the 2/4/02 submission be returned as sketches).
22-Feb-02	USDA approved labels submitted on 02/04/02 as sketches (per FDAH's request on 2/21/02).
25-Feb-02	Requested to ship up to 12 doses each of Serials 1749-20-122801 and 1749-25-122801 to Saitama, Japan.
13-Mar-02	USDA gave permission (verbal perm. given on 3/7/02) to ship up to 12 doses each of Serial Nos. 1749-20-122801 and 1749-25-122801 to Dr. Setsuo Arai of the Kitasato Institute, Saitama, Japan.
14-Mar-02	The Biological Product License was issued (we rec'd on 3/15/02).
14-Mar-02	USDA approved labels submitted on 2/21/02.
20-Mar-02	Submitted FDAH's press release to USDA.
21-Mar-02	Faxed a revised press release per telephone conversation between Dr. Ludemann & Dr. Steve Chu on 3/20/02.
22-Mar-02	USDA approved FDAH's press release subt'd on 3/20/02. This also confirms USDA's verbal approval on 3/21/02 (faxed) to add the patent acknowledgements for the University of California and the University of Florida.
22-Mar-02	Submitted efficacy report in 8-week-old kittens. (Cover ltr dated 4/1/02)
5-Apr-02	Submitted for USDA approval a press release to be distributed internationally.

## BRIEF DESCRIPTION OF ACTIVITIES DURING REGULATORY REVIEW PERIOD FOR FIV

Updated 5/8/2002

Date	Description
18-Apr-02	USDA approved the press release to be distributed internationally.
24-Apr-02	Submitted a quick synopsis on the FIV 6-month DOI. No response from USDA is required.
26-Apr-02	Submitted 2 separate letters ("Dear Distributor:" and "Dear Animal Health Industry Professional:") with advertising material attachments for USDA review and approval.
1-May-02	USDA gave verbal authorization (also confirmation letter dated 5/1/02) to distribute the advertising material identified as 2 letters.

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